ABSTRACT

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CELLULAR AND MOLECULAR BASIS OF IMMUNITY AND PATHOGENESIS OF *CHLAMYDIA TRACHOMATIS*

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The purpose of this study was to examine the influences of estrogen and chemokine receptor-5 (CCR5) on immune response and Chlamydia pathogenesis. *Chlamydia trachomatis* is the most common bacteria that causes sexually transmitted disease (STD) worldwide. Clearance of chlamydia infections is due to a robust T cell (Th1) response. Immunological and hormonal factors such as 17β-estradiol and chemokine and/or chemokine receptors may influence the induction of protective immunity against chlamydia infections. In fact, estrogens such as 17β-estradiol could influence the genital mucosal immunobiology, including cytokine expression and recruitment and trafficking of leukocytes into the reproductive tract.

Genetically engineered knockout animals, molecular and immunological techniques were used to test the hypothesis that estrogen and CCR5 play pivotal roles in the pathogenesis and immunity against chlamydia infection by modulating the chemokine production and Th1 response.

Results indicated that in 17β–estradiol treated cells, there was an enhancement of chlamydia inclusion bodies. Moreover, there was a decrease in the production of Th1 chemokines [regulated activated normal secreted and expressed T cells (RANTES) Macrophage Inducible Protein-1α/β (MIP-1α /MIP-1β), interferon-inducible protein 10 (IP-10)]. Delayed clearance of genital infection was observed in wild-type mice that were pretreated with estrogen. There was no comparable effect on the fertility (measured by pregnancy rate) of estrogen treated mice during chlamydia infection and that of untreated infected mice.

When genitally-infected wild-type (WT) and CCR5 knockout mice were evaluated for microbiologic shedding of chlamydiae, there was a greater intensity of infection and delayed resolution in the knockout mice. However, compared to the WT mice, the fertility of infected CCR5KO mice was only mildly affected in the short term and unaffected in the long term. In addition, immunobiologic analysis revealed that the diminished capacity of CCR5KO to control acute chlamydial infection correlated with the relatively low chemokine and cytokine expression corresponding to a poor early Th 1 response. However, reduced incidence of complications in the CCR5KO mice appears to correlate with the low activity of long term inflammatory mediators.

The findings of this study may be helpful in the development of therapeutic and/or preventive treatments to control the onset of genital chlamydia associated complications.

CELLULAR AND MOLECULAR BASIS OF IMMUNITY AND PATHOGENESIS OF *CHLAMYDIA TRACHOMATIS*

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LIST OF ABBREVIATIONS

µg	microgram
µl	microliter
2-ME	2-mercaptoethanol
ANOVA	analysis of variance
APC	antigen presenting cells
CCR5	Chemokine Receptor-5
$CCR5^{-/-}$	chemokine receptor-5 Knockout
CDC	Centers for Disease Control
CMI	Cell mediated immune response
CO_2	carbon dioxide
CTLs	Cytotoxic T lymphocytes
CX	Cycloheximide
CXCR3	Chemokine Receptor-3
DNA	Deoxyribose nucleic acid
EBs	Elementary bodies
ELISA	Enzyme-Linked Immunosorbent Assay
FITC	fluorescein isothiocynate
FSH	Follicle stimulating hormone

LIST OF ABBREVIATIONS

GM-CSF	Granulocyte Macrophage-Colony Stimulating Factor
GnRH	gonadotropin-releasing hormone
ICAM-1	intracellular adhesion molecule
IDO	Indoleamine 2, 3-di-oxygenase
IFN-γ	Interferon-gamma
IFU/ml	inclusion-forming units per milliliter
IgG	immunoglobulin G
IL-12	Interleukin-12
IL-13	Interleukin-13
IgE	immunoglobulin E
IL-1α	interleukin-1α
IL-4	Interleukin-4
IL-5	Interleukin-5
iNOS	inducible nitric oxide synthase
IP-10	interferon-inducible protein 10
KCL	Postassium Chloride
kd	kilodalton
LFA-3	leukocyte function antigen type-3
LGV	lymphogranuloma venereum

LIST OF ABBREVIATIONS

LH	lutenizing hormone
LPS	lipopolysaccharide
MCP-1	monocyte chemotactic protein-1
mg/ml	milligram per mililiter
$MgCL_2$	Magnesium Chloride
MHC II	major histocompatibility complex class II
ml	mililiter
mM	milimolar
MOMP	major outer protein
MoPn	mouse pneumonitis
NH_4Cl	Tris-Ammonium Chloride
nm	nanomolar
NO	nitric oxide
°C	degrees Celsius
PBS	Phosphate-Buffered Saline
pH	power of Hydrogen
PID	pelvic inflammatory disease
PMN	polymorphonuclear
RANTES	regulated on activation, normal T cell expressed and secrete

LIST OF ABBREVIATIONS

RB	reticulate body
RNA	ribose nucleic acid
RPMI	Roswell Park Memorial Institute
RT-PCR	Real-Time Polymerase Chain Reaction
STD	Sexual transmitted disease
TCR	T cell receptor
Th1	T helper type 1
Th2	T-helper type 2
TNF- α	Tumor necrosis factor-alpha
WHO	World Health Organization

CHAPTER 1

INTRODUCTION

The family Chlamydiaceae consists of one genus *Chlamydia* with three species that cause human disease *C. trachomatis, C. psittaci, and C. pneumoniae*. *C. precorum* has not been associated with any human disease. Chlamydiae are small obligate intracellular parasites which were previously considered to be viruses. However, they contain deoxyribose nucleic acid (DNA), ribonucleic acid (RNA) and ribosomes for making their own proteins and nucleic acids and now considered to be true bacteria. They possess an inner and outer membrane similar to Gram-negative bacteria and a lipopolysaccharide, but do not have a peptidoglycan layer. Although they synthesize most of their metabolic intermediates, they are unable to make their own adenosine triphosphate and thus are energy parasites. Chlamydial infectious agents have a tropism for mucosal epithelial cells of the eyes, lungs, and reproductive tract, where they many times exist in an asymptomatic state with the host cell acting as a natural reservoir for the bacterium (Mather 1980; van Westereenen *et al.* 1998; Igietseme *et al.* 1996). Transmission can occur through direct contact and aerosol, and requires no alternate vector, although infections can be transmitted from flies to ocular regions in trachoma endemic areas (Sheff 2000).

Chlamydia trachomatis is the cause of the most widely spread bacterial sexually transmitted disease (STD) worldwide. The World Health Organization (WHO) recently

revealed that *C. trachomatis* infections account for more than 90 out of 500 million annual new STDs worldwide. In 2004, 929,462 cases were reported to the Centers for Disease Control (CDC) from 50 states and the District of Columbia (CDC 2004). The pathological consequences of genital infection by *C. trachomatis* in women are severe sequelae associated with ascending infection including pelvic inflammatory disease (PID), fallopian tube scarring, ectopic pregnancy and infertility. The frequent asymptomatic nature of Chlamydia infections often result in the presentation of these complications as the first evidence of infection. In men, the initial stage of genital chlamydial infection involves inflammation of the urethra such as non-gonococcal urethritis. Untreated cases result in men experiencing fever, swelling and pain in the scrotum; symptoms of acute epididymitis, which is inflammation of the sperm ducts ventral to the testicles. Presently, there is no evidence that this pathogen causes infertility in men (Krause and Bohring 2003).

Chlamydial infections are treatable with antibacterials, such as tetracycline derivatives, especially doxycycline, and the macrolides or azalides such as erythromycin and azithromycin; however, as previously mentioned, infections are often asymptomatic, with severe complications usually presenting as the first symptoms of an infection (Igietseme 2002). The dilemma posed by chlamydial infections in both the developed and developing nations has intensified efforts to design preventive and control measures, of which frequent screening for early detection and treatment, and the administration of an efficacious vaccine have become a priority (Igietseme 2002). However, there are obvious difficulties in establishing acceptable and cost-effective, community-wide

screening programs or rational antibacterial prophylaxis. Therefore, timely diagnosis using contemporary methodologies, and application of chemotherapy to arrest silent or persistent infections have not been established to control chlamydial infections. This situation has resulted in the deep concern that chlamydial infections may pose a serious threat to human reproduction, longevity and general health quality, as well as constituting a considerable burden on national healthcare budgets and management. A vaccine is the approach to delivering long-lasting protection to the largest number of people worldwide. In fact, computer modeling has indicated that even a partially successful vaccination program would have a remarkable global impact in reducing chlamydial infections, disease prevalence and related expenditure (Moore *et al.* 2002). Therefore, while the ultimate goal of a chlamydial vaccine is ideally to achieve sterilizing immunity, a vaccine effective against disease sequelae, such as PID, blinding trachoma and tubal scarring would be acceptable as a first-generation product. A better understanding of the immunobiology of chlamydial infections will be crucial for vaccine design. Although many advances have been made in chlamydia research, limited progress has been made in defining the immune effectors that mediate chlamydia immunity.

Animal model studies have revealed that mice infected intravaginally with mouse pneumonitis biovar of *C. trachomatis* (MoPn) develop a self-limiting infection, which generally resolved in 18 to 21 days (Barron *et al.* 1981). Genital inoculation of mice with MoPn initiates infection in the vaginal epithelium and ascends the epithelial surface of the uterine horns and oviducts (Morrison *et al.* 1995). Infection is usually confined to the genital tract mucosal epithelium. The host response occurs in two phases which involves the secretion of chemoattractant proteins referred to as chemokines from the

infected cell such as dendritic cells, macrophages and epithelial cells. These chemokines include regulated on activation, normal T cell expressed and secreted (RANTES), monocyte chemoattractant proteins (MCP-1), and macrophage inflammatory protein-1 alpha and beta (MIP-1α, 1β) (Belay *et al.* 2002). They elicit a specific immune response by stimulating the activation and migration of T and B cells.

The profile of immune responses measured following chlamydial infection of humans and experimental animal models include a strong T cell response characterized by chlamydial specific $CD4^+$ and $CD8^+$ T cells and their cytokines, which are induced following exposure to Chlamydia (Igietseme 1996). Cell mediated immune (CMI) response involving Th1 cells has been found to be crucial for clearance of a genital chlamydial infection (Stagg 1998; Bose *et al.* 1986; Moore *et al.* 2002; Paavonen 1989). The importance of CMI in protection against chlamydial genital tract infection was first demonstrated with T-cell deficient athymic (nude) mice and mice depleted of $CD4^+$ T cells (Morrison *et al.* 2002). Chlamydiae-specific $CD4^+$ or $CD8^+$ T cell lines and clones also impart partial protection to naïve recipient mice, however, the magnitude of protection conferred by $CD4^+$ T cells is distinctly superior to that by $CD8^+$ T cells. Collectively, many lines of evidence strongly implicate $CD4^+$ T cells as an important lymphocyte subset in mediating antichlamydial immunity. The Th1 cytokine interferon gamma (IFN-γ) is essential for optimal clearance of infection from genital tract tissue (Morrison *et al.* 2002). The mechanisms by which IFN- γ could inhibit chlamydial intracellular growth are numerous, due to its pleiomorphic effects on host cell function. The two IFN- γ inducible host cell functions that have received the majority of attention in studies of chlamydial immunity are the induction of inducible nitric oxide synthase and

of tryptophan-decyclizing enzyme indoleamine 2,3-dioxygenase (Igietseme 2002). The production of bactericidal nitric oxide synthase resolves both primary and secondary chlamydial infections. Conversely, the depletions of intracellular tryptophan pools by indoleamine 2, 3-dioxygenase is inhibitory to chlamydial growth due to the parasites' tryptophan auxotrophy (Morrison *et al.* 2002). The sensitivity of human strains of chlamydia to IFN-γ indicates that stimulation of mucosal IFN-γ Th1 responses play a key role in the eradication of infection (Morrison *et al.* 2002). Therefore, it would be valuable to gain better insight of the factors that may regulate the induction, recruitment, and persistence of Th1 cells. In general, these factors that may regulate the T cell processes are antigen presenting cells (APCs), cytokines/chemokines released by infected cells, cytokine/chemokine receptors, and the hormonal environment during infection.

Belay *et al.* (2002) demonstrated that Chemokine Receptor-5 (CCR5) was highly expressed on T-cells in the genital tract 21 days post chlamydial infection. The chemokine receptor-5 (CCR5) acts as a co-receptor with the $CD4^+$ molecule for the entry of human immunodeficiency virus (HIV-2) into host cells (Huang *et al.* 1996; Dean *et al.* 1996). Interestingly, chlamydia has been associated with an increased risk of human immunodeficiency virus-related AIDS (Morrison *et al.* 2002; Anttila *et al.* 2001; and Oshige K.S. *et al.* 2000). The chemokine receptor CCR5 is a member of the 7-transmembrane, G protein-coupled receptor superfamily, functioning as an important chemokine receptor that is preferentially expressed on certain leukocytes (monocytes, cytotoxic T cells, and CD4, T-helper 1 (Th1) and dendritic cells, and binding specific chemokines (RANTES, MIP-1α, MIP-1β) that activate and induce Th1 cells. As a crucial receptor involved in T cell activation and function, a deficiency of CCR5 is

associated with a suppression of T cell induction and leukocyte migration under certain infectious and non-infectious conditions, suggesting that it plays a role in both infection-related immune and inflammatory processes (Barr *et al.* 2005). The effect of a targeted suppression of the critical specific T cell response on both immune-mediated microbial clearance and the development of complications of chlamydial infection is largely unknown.

Epidemiologic evidence suggests that infection with *C. trachomatis* is enhanced by oral contraceptives (Washington *et al.* 1985). This link between hormonal contraception and cervical infections is biologically plausible because estrogen and progesterone can enhance or suppress the growth and persistence of vaginal flora (Kaushic *et al.* 2000). In a quantitative review by Cottingham and Hunter (1992), it was observed that women who chose Depo-Provera as a contraceptive experienced a three fold increase in the risk of acquiring two of the most commonly spread bacterial STDs: chlamydia and gonorrhea. Moreover, estrogen pretreatment has been associated with increased chlamydia growth using *in vivo* and *in vitro* models (Rank 1989; Bose *et al.* 1999; Guseva 2003). The exact cause for this enhancement is yet to be studied. There is a lack of knowledge on the effects of hormones on the cellular and molecular effectors of chlamydia immunity.

The purpose of this study was to investigate the influence of estrogen, and the Th1 receptor CCR5 on the pathogenesis and immunity against genital chlamydia infection. Accordingly, both *in vitro* and *in vivo* studies were used to investigate the role of 17 β-estradiol and chemokine receptors on T cell activation, and protection from complications during genital chlamydia infection. The following specific aims were used to test the

hypothesis stating that both estrogen and CCR5 play pivotal roles in the pathogenesis and immunity against chlamydia by modulating the chemokine production and Th1 response:

1) **To determine the influence of estrogen on the pathogenesis and immune response against genital chlamydia infection**

   1a. Investigate the effects of estrogen on chlamydia infectivity and chemokine expression during infection of epithelial cells

   1b. Evaluate the effects of estrogen on the course of infection and chemokine production during genital chlamydia infection in mice

   1c. Determine the effects of estrogen on fertility of chlamydia infected mice

2) **To investigate the influence of CCR5 on the pathogenesis and immune response against genital chlamydia infection**

   2a. Assess the effect of CCR5 on the course of infection and chemokine/cytokine response in chlamydia infected mice

   2b. To evaluate the effects of CCR5 on fertility genital chlamydia infected mice

This study has revealed novel findings indicating that estrogen and CCR5 have key roles on the immune response during chlamydia infection. This is the first study to show the effects of estrogen and CCR5 on inflammatory cytokine response during genital chlamydia infection. These findings may be helpful in developing therapeutic treatments protecting infected women from the development of inflammation that is associated PID.

CHAPTER 2

REVIEW OF LITERATURE

Structure and Characteristics of Chlamydia

Chlamydia primary antigens include the 45 kilodalton (kd) glycoprotein major outer protein (Omp 1 or MOMP), 60 kd cysteine rich protein (OMP2) and porB, a low molecular weight protein. Ultrastructurally, chlamydiae are complex and display attributes not found in other bacteria. The envelopes of both elementary bodies (EBs) and reticulate bodies (RBs) have two trilaminar membranes, an outer membrane and an inner cytoplasmic membrane (Stephens 1999); in this respect chlamydiae are similar to other Gram-negative bacteria. Chemical evaluations have demonstrated that chlamydiae lack muramic acid and thus do not contain peptidoglycan. Another unique ultrastructural feature of this pathogen is the surface projections that extend from the cytoplasmic membrane and protrude approximately 20 nm above the outer membrane. The outer membrane complex components defined at the molecular level are LPS, MOMP, OMP2, and OMP3. Both LPS and MOMP on RBs are surface-accessible by antibodies. OMP2 and OMP3 are developmental stage-specific cysteine-rich proteins present only in EBs (Stephens 1999). *Chlamydia trachomatis* is a strict pathogen of oculogenital epithelial cells. It is the etiologic agent of trachoma and is the leading cause of bacterial sexually transmitted diseases (STDs) worldwide (Brunham 1999, WHO 1996). *C. trachomatis*

and *C. psittaci* has several serovars with each species that differ in cell wall composition and the presence of particular outer membrane proteins (Rank 1999). *C. trachomatis* isolates consist of 15 major serovariants that can be distinguished among clinical isolates, based on serological distinction of variation in the major outer membrane protein (MOMP-a translation product of ompA) (Stagg 1998). Recently, genotypic identification and confirmation of these isolates by ompA-based nucleic acid amplification and sequencing methods have led to an alternative designation, genovar or genotype. The classification is useful in pathologic, epidemiologic and detailed taxonomic analysis of chlamydiae and their evolution in the microbial realm (Igietseme 2002; Morrison *et al.* 2002; Loomis *et al.* 2002; Brunham 1999). The closely related murine strain designated MoPn was recently reclassified as *C. muridarum* (Everett, *et al.* 1999). Of the 15 odd serovars of *C. trachomatis*, serovars A, B, Ba, and C cause trachoma. Serovars D to K and the lymphogranuloma venereum (LGV) strain L1, L2 and L3 cause sexually transmitted genital infection in the human populations. This infection has been associated with causing cervicitis, urethritis and complications linked to more severe disease such as endometritis, salpingitis and pelvic inflammatory disease (PID). LGV and D to K serovars differ biologically and pathologically. Serovars D to K are noninvasive and may cause infection and disease that are restricted to the urogenital mucosae (Morrison 2002). The lymphogranuloma venereum infections are invasive and often ulcerative with lymphatic tissue involvement (e.g. inguinal bubo) (Yang 1998; Patton 1992). These infections are endemic in certain developing nations, including parts of Africa, Asia, South America, and the Caribbean (Rank 1999). Lymphogranuloma venereum transiently infect epithelial cells, invade the submucosae to infect

macrophages, the macrophages then facilitate the dissemination of infection to regional draining lymph nodes.

In fact, genital infections by the different genovars of *C. trachomatis* constitutes the most common bacterial STD in the United States and several other industrialized nations, including the United Kingdom, Germany, Japan, and France. The World Health Organization (WHO) recently revealed that *C. trachomatis* infections account for more than 90 of 500 million annual new STDs worldwide. The United States alone spends over $2 billion annually on four million reported cases (WHO 1996, CDC 2004); this constitutes an enormous morbidity and socioeconomic burden of chlamydial infections. Moreover, reports suggesting that genital chlamydial infection may be on the rise (Igietseme 2002), and could predispose to HIV-related AIDS (Cohen 2000; Cohen 1999) and human papilloma virus-associated cervical dysplasia, have heightened these concerns (Gopalkrishna *et al.* 2000; Hakama *et al.* 2000).

Chlamydia pneumoniae (also known as *Chlamydophila pneumoniae*) was previously known to cause mild to sub-lethal acute respiratory infections that may on occasion progress to pharyngitis, bronchitis and even pneumonia. The pathogen has recently been associated with atherosclerosis, adult-onset asthma and certain other chronic diseases (Igietseme 2002; Martson 2002; Rajalingam 2001). The zoonotic *C. psittaci* constitutes an occupational hazard for workers in the poultry and farming industry, and persons exposed to infected avian species (Buzoni-Gatel 1992). In animal and birds, *C. psittaci* infections cause psittacosis, hepatitis, mastitis, conjunctivitis, pneumonia, abortions and diarrhea, but in humans the infection causes a psittacosis-like disease that may on rare cases become systemic and fatal. Finally, the fourth species of

Chlamydia, *C. pecorum*, has not been associated with any human disease (Brunham 1999).

The developmental cycle of Chlamydia is characterized by an alternation between two structurally and physiologically distinct forms: the infectious elementary body (EB) and the non-infectious replicative reticulate body (RB).

The EB possesses a rigid outer membrane that is extensively cross-linked by disulfide bonds. Because of their rigid outer membrane EBs are resistant to harsh environmental conditions encountered when the chlamydia are outside of their eukaryotic host cells. The metabolically active and vegetative intracytoplasmic RB possesses a fragile membrane lacking the extensive disulfide bonds characteristic of the EB.

Chlamydial infection is initiated by the attachment via receptors to a susceptible host cell of the EB. The EBs are endocytosed by epithelial cells. EBs become metabolically active, and replicate in modified vacuoles, termed inclusions. The EB is rapidly transformed into a RB, which replicates by binary fission within the confines of the inclusion. As the developmental cycle progresses, progeny Chlamydia condense into infectious elementary bodies that are released by exocytosis or host cell lysis (Fig. 1).

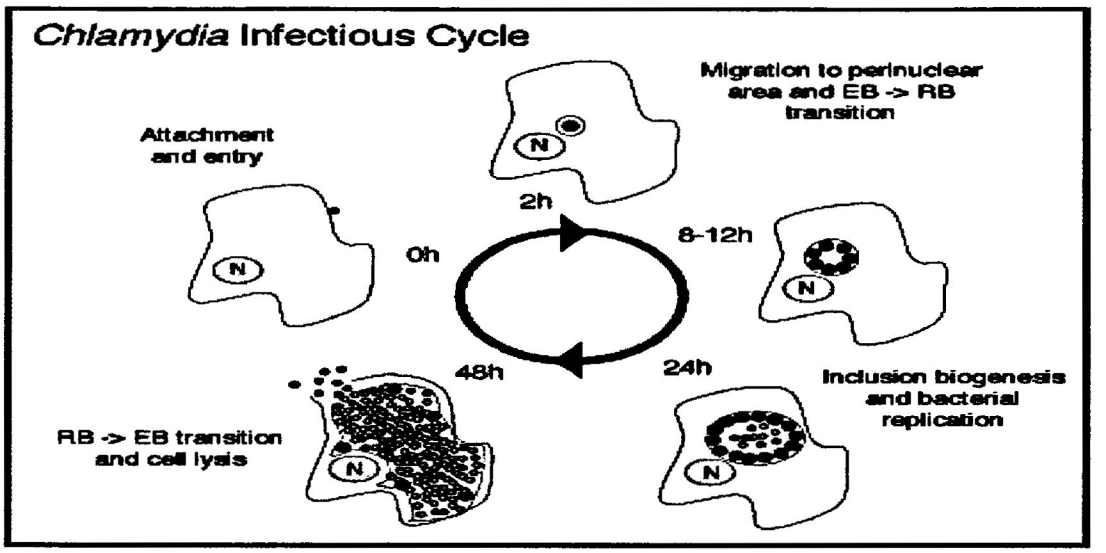


Figure 1. Chlamydia Developmental Cycle (used by permission)

Host Immune Response during Genital Chlamydia Infection

Different animal models have been developed to study the pathogenesis and host immune response to *C. trachomatis* infection as a prerequisite for accumulating information to guide the design of prevention strategies, including an effective vaccine against chlamydia. Of these animal models, most information, both in terms of pathologic features and immunobiology of the genital infection, has emerged from experimental infections and immunization studies in mice, guinea pigs and monkey (Patton *et al.* 1992). Perhaps the best-defined model of immunity of *chlamydiae* developed to date is the mouse genital tract infection system using murine or human isolates of *C. trachomatis* (Morrison 2000; Patton and Rank 1992). In addition to the availability of information and reagents for detailed pathologic and immunologic analyses, the mouse model of chlamydial genital infection exhibits disease patterns that are similar to human disease (Morrison *et al.* 2002). The pathologic features include

chlamydial shedding, uterine and tubal inflammation, and development of complications such as hydrosalpinx, and tubal factor infertility (Igietseme 2003). Furthermore, the elements of protective anti-chlamydial immunity identified in mice appear to be relevant for chlamydial control in humans (Brunham 1999). The mouse model of chlamydial genital infection has therefore yielded and continues to furnish highly useful pathologic and host response information in the areas of disease evolution, the essential elements of protective immunity and the basis for vaccine selection and evaluation that could be extrapolated to humans.

The immune system maintains a significantly different response to intracellular and extracellular pathogens (Lagrange *et al.* 1985, Deshpande *et al.* 2000). The goal of the immune response to extracellular pathogens involves eradicating the pathogen itself and neutralizing its infectious products. Antibodies that are particularly effective against extracellular pathogens are more complex and involve several options for disease elimination. An immune response to intracellular invasion should aim to either directly destroy the infected host cell through cytotoxic mechanisms or should act to activate infected target cells to destroy the pathogen itself (Patton *et al.* 1992). Therefore, clearance of an established intracellular infection involves the elicitation and activation of T cells and/or natural killer cells, as well as other effectors of T cell-mediated immune response.

Pathogens, such as *C. trachomatis*, are controlled by an assortment of immune effectors. An important element in the development of a reliable prophylaxis to control Chlamydia, such as a vaccine, is the clarification of immunologic and other factors that will ensure long-term immunity in order to achieve better vaccine efficacy. The cellular

immune response to chlamydial infection is characterized by complex interactions between many different effector cells responding to the pathogen (Igietseme 2002; Loomis 2002). Chlamydial genital disease initiates at and appears to be confined to the epithelium of the genital mucosae (Hodinka 1998; van Westereenen *et al.* 1998). Specific lymphoepithelial interactions in the genital mucosae are associated with genital exposure to *chlamydiae.* These interactions lead to the elicitation of anti-chlamydial immune responses, with resultant development of specific immunity and resolution of disease.

A host response to chlamydial infection is two-fold. The host must protect itself against both the infectious EBs colonizing new cells and those residing in previously infected cells. Inflammation is the hallmark of chlamydial infection and involves a focal inflammation marked by high infiltration of polymorphonuclear cells (PMNs or neutrophils) as an initial immune response. This is followed by mononuclear cells, which are recruited to the site of infection. Subsequently, specific immune responses involving T and antibody-secreting B cells are elicited (Loomis 2002; Morrison *et al.* 2002). Thus, the profile of immune responses measured following chlamydial infection of humans and experimental animal models include a strong T cell response characterized by chlamydial-specific $CD4^+$ and $CD8^+$ T cells, and their cytokines which are induced following exposure to Chlamydia (Morrison 2000; Igietseme and Rank 1991; Moore *et al.* 2002). In addition, humoral immune effectors characterized by activated EB cells, serum and secretory anti-chlamydial antibodies are elicited during a genital chlamydial infection (Moore *et al.* 2002).

Host immune responses during genital chlamydia infection are directed at different chlamydial antigens, but the level of contribution of antibodies versus T cells to chlamydial clearance and establishment of immunity requires clarification in order to better target vaccines.

Role of Th1 Effectors in Chlamydial Clearance and Immunity. Cell mediated immune response (CMI) involves the induction of T helper type 1 cells (Th1), cytotoxic T lymphocytes (CTLs) and their cytokines, which are capable of activating other inflammatory cells such as macrophages, NK cells, and dendritic cells. The $CD4^+$ T helper cells recognize foreign antigens via a peptide complexed to major histocompatibility complex class II (MHC II) molecules on dendritic cells, macrophages, and other antigen-presenting cells. Upon activation, $CD4^+$ T helper cells are the primary regulators of several T and B cell mediated responses including: 1) providing help for antigen-stimulation of subsets of B cells to proliferate and produce antibodies of different isotypes and 2) activating other effector T cells in cell-mediated immunity (Loomis 2002; Darville 2006).

$CD4^+$T cells can be subdivided into different subsets. The differentiation process is initated by the ligation of the T cell receptor (TCR); and cytokines present during the initiation of a T cell response determine the development of the particular T helper type 1 (Th1) subset (Bonnechi, 1998). T-helper type 1 cells secrete IL-12, IFN-γ and lymphotoxin, whereas T-helper type 2 (Th2) cells secrete IL-4, -5, and -13. T-helper type 1 cells predominately control cell-mediated immune responses and appear to be involved in chronic inflammatory conditions, whereas Th2 cells upregulate immunoglobulin E (IgE) production and are prominent in the pathogenesis of allergic diseases (Bonnechi

1998). Polarization of the T cell subsets most likely occurs in the secondary lymphoid organs to which naïve T cells preferentially migrate. Memory lymphocytes and effector precursor cells, in contrast, migrate to peripheral tissues. It is likely that, given their different effector functions, Th1 and Th2 cells are differently recruited to peripheral sites of inflammation (Baggiolini 1997). Indeed, it has been shown that Th1, but not Th2 cells, express a functional ligand for P- and E-selectin and therefore are selectively recruited to sites where Th1 immune responses occur (Bonecchi *et al.*1998). The lymphocytes include the B cells, $CD4^+$ and $CD8^+$ T cells (Morrison *et al.* 2000).

Analyses of hosts antichlamydial immune mechanisms in animal models have shown that in guinea pigs both humoral and cell mediated immunity are essential for the resolution of and resistance to infection (Rank *et al.* 1985). Studies using athymic nude mice revealed the importance of cell-mediated immunity in protection against chlamydial genital tract infection. In this study performed by Rank and colleagues (1985), 8 week old nude mice (*nu/nu*) and mice heterozygous for the nude trait *(+/nu*) were inoculated intravaginally with mouse pneumonitis (MoPn), the heterozygous mice resolved their infection in 20 days and had an increased titer of immunoglobulin G (IgG) antibody to MoPn by day 17, whereas the homozygous mice showed a decrease in IgG and developed chronic infection which lasted 265 days (Rank *et al.* 1985). Moreover, adoptive transfer experiments have confirmed the importance of $CD4^+$ T cells in the clearance of Chlamydia (Ramsey *et al.* 1991; Su *et al.* 1995).

In addition to $CD4^+$ T cells, $CD8^+$ cells also contribute to antichlamydial immunity *in vivo (*Igietseme *et al.* 1994). The mouse model demonstrates a subordinate role for CD8+ T cells in the resolution of genital infection. Mice deficient in β2-

microglobulin resolve infection as efficiently as wild-type mice (Darville 2006), and mice deficient in the cytolytic effector molecules, perforin and CD95 (Fas), effectively clear infection with *C. muridarum*. However, *C. muridarum*-specific $CD8^+$ T cells efficiently lyse *C. muridarum*-infected cells transfected with intracellular adhesion molecule ICAM-1, indicating the potential for $CD8^+$ T cells to contribute to the elimination of infection. Gene knockout studies have indicated that $CD8^+$ and B-cell knockout mice are as potent as immunocompetent wild-type mice (Johansson *et al.* 2001, 1997; Morrison *et al.* 1995; Su *et al.* 1995) in the clearance of chlamydia. These results suggest that neither B cells, antibodies, nor $CD8^+$ T cells contribute exclusively in the clearance of primary chlamydial infection. In contrast, $CD8^+$ cell deficient mice remain protected from re-challenge (Morrison *et al.* 2000), whereas B-cell deficient mice are susceptible to re-infection and exhibit delayed clearance of chlamydiae, suggesting that B cells may play an important role in resistance to re-infection.

Role of antibodies in induction of protective immunity against *C. trachomatis.* The mouse model suggests a lack of importance for B cells or antibody in resolution of primary genital infection, as B cell deficient mice resolve primary infection normally, and passive immunization has a minimal effect on the course of primary infection. In the female guinea pig model of genital infection however, antibody plays an important role in eradication of infection. Antibody-depleted guinea pigs maintain a high level of primary infection, and passive immunization of naïve animals results in a marked decrease in the level of primary infection compared with controls. Moreover, Moore *et al.* (2002) showed that B cells participate in anti-chlamydial immunity via FcR-mediated effector function of antibodies, which are operative during re-infections.

Antibodies specific for *C. trachomatis* can neutralize infection in tissue culture. However, high titer of *C. trachomatis*-specific antibody does not correlate with increased severity of sequelae, such as tubal factor infertility (TFI) (Morrison 2002). In these epidemiological studies, high titers of antibody may reflect repeated infection, known to be associated with increased morbidity. Although antibody may not play a primary role in resolution of infection, studies suggest it may help control the shedding of organisms and protect against upper tract disease. One study reported the prevalence of mucosal IgA antibodies was inversely related to the quantity of C. *trachomatis* shed from the human endocervix (Darville 2006), and another found the presence of serum IgA and IgG antibodies reduced the risk of ascending infection among women undergoing therapeutic abortion. Ramsey *et al.* (1988) characterized the humoral immune response to MoPn genital infection. By enzyme-linked immunosorbent assay, they found that IgG (Immunoglobulin G) first appeared in the plasma on day 14 and that IgA (immunoglobulin A) was first detected in vaginal washes on day 21. However it has also been found that mice are capable of resolving MoPn genital infections in the absence of antibody response (Ramsey *et al.* 1988). Interestingly, it was also observed from a similar study that mice were susceptible to re-infection, which could possibly mean that the immune response lowers significantly to allow the establishment of a second infection. In addition, Igietseme and Rank (1991) concluded that susceptibility to re-infection was due to the intensity of the local T cell mediated immune response in the genital tract.

Role of Co-Stimulatory and Adhesion Molecules in Chlamydial Clearance and Immunity.

Certain co-stimulatory and adhesion molecules expressed by antigen presenting cells

(APCs) are important for effective immune activation and maintenance and so their modulation could lead to up-regulation of the required Th1 response during vaccine delivery against Chlamydia. For instance, dendritic cell (DC) potency as high efficient APCs is due in part to their high co-stimulatory ability, which is associated with the elevated density expression of co-stimulators such as the B7, CD40, ICAM-1 and leukocyte function antigen type-3 (LFA-3) molecules (Jenkins *et al.* 1993; King *et al.* 1993). Both co-culture experiments and *in vivo* work support role for the ICAM-1 in anti-chlamydial immunity (King 1993). Intracellular adhesion molecule-1 is a cell adhesion molecule expressed by leukocytes, endothelial and epithelial cells. It is a prominent member of the immunoglobulin super gene family of proteins that function as adhesions in host defense and pathology. ICAM-1 plays roles in rapid T cells activation, recruitment, and retention in the genital mucosa of infected animals by binding with high affinity to LFA-1 on the surface of T cells. Following binding, ICAM-1 is involved in signal transduction for lymphocyte activation and function (Freyer 1999). $ICAM^{-/-}$ mice displayed a delayed Th1 response to chlamydial genital infection, severe acute cervical and ascending infection, and high rates of tubal complications (i.e. hydrosalpinx), which is associated with a slow activation of specific Th1 cells by ICAM-1 $^{-/-}$ APCs (Freyer 1999).

Additional co-stimulatory molecules include CD40, which belong to the TNF-family of receptor proteins and plays a central role in the effector function of fully differentiated T cells. CD40 is expressed on APCs and interacts with the CD40 ligand on the T cell's surface to contribute to bi-directional signaling, where both the T cell and the APC receive activating signals (King *et al.* 1993). CD11c is a component of the

leukocyte integrin (CD11c/CD18 or CR4) on mature dendritic cells, functioning in leukocyte adhesion and phagocytic responses (Serre 1998). The co-stimulatory B7 molecules, B7.1 (CD80) and B7.2 (CD86), are members of the immunoglobulin gene super family of proteins and the principal co-stimulatory molecules expression on APCs. The homodimers bind to CD28 on the surface of T cells and clonal expansion of T cells cannot occur until this binding takes place (Jenkins *et al.* 1993; King *et al.* 1993). B7.1 (CD80) and B7.2 (CD 86) help $CD4^+$ T precursor cells to mature along two alternative pathways, Th1 and Th2, respectively. On APCs, the interactions of B7.1 and B7.2 have shown to be reciprocally expressed with the down-regulation of the one leading to concomitant up-regulation of the other. Interestingly, their roles have been demonstrated both *in vitro* and *in vivo* to be predominant in altering initial cytokine profiles of activated cells rather than overall cell induction (Kelly and Rank 1997).

Influential factors in the Induction of Protective Immunity

Since it has been established that CMI effectors are required for protective immunity against genital chlamydia infection, a better understanding of the factors that influence the generation of optimal T-cell response during infection is important for the development of an effective vaccine and/or therapeutic agents. In this respect it appears that factors that may regulate the T cell processes APCs, cytokines/chemokines released by infected cells, cytokine/chemokine receptors, and the hormonal environment during infection.

Cytokine/Cytokine Receptors. Chemokines, a superfamily of polypeptide mediators, are a key component of the leukocyte recruitment process (Bonecchi *et al.* 1998). The relative position of a Cys tandem defines four structural motifs (CXC, CC, C

and CX3C). Five receptors for CC chemokines (named CCR1 through 5) and four for CXC (CXCR1 through 4) have been defined. Increasing evidence suggests that chemokines play an important role in the regulation of T-helper type 1 (Th1) and T-helper type 2 (Th2) responses *in vivo*. The induction of specific Th1-recruiting chemokines during primary and secondary genital chlamydial infection has been established with protective Th1 cells recruited to infected genital mucosa more efficiently under the influence of specific chemokines (i.e. RANTES, MIP-1α and MIP-1β) than non-protective Th2 cells (Kelly and Rank 1997).

Chlamydia infection generates a cytokine response both by direct infection of epithelial cells lining the moist surfaces of the body and interaction with cells of the immune system (Fitzpatrick 1991). Infection of epithelial cells derived from human cervix, a major cause of the genital tract infection generates various cytokines such as [(interleukin-6 (IL-6); interleukin-8 (IL-8), interleukin-1α (IL-1α)], (Granulocyte Macrophage-Colony Stimulating Factor, (GM-CSF). Belay *et al.* (2002) demonstrated that after genital chlamydia infection in C57 mice, elevated levels of chemokines RANTES, monocyte chemotactic protein-1 (MCP-1), macrophage inflammatory protein 1α (MIP-1α), and gamma-interferon-inducible protein 10 (IP-10) were produced. RANTES, MCP-1, MIP-1α and IP-10 play significant roles in leukocyte activation and recruitment during Chlamydia infection (Figure 2) These studies were in accord with other findings by Darville *et al.* (2001), who examined TNF-α, IL-1β IFN-γ, IL-10, (macrophage inflammatory protein-one alpha) MIP-1α, and (monocyte chemoattractant protein) MCP-1 in various strains of mice (C57BL/6, BALB/c, and C3H). It was

concluded that there was a prolonged and increased levels of TNF-α, IL-1β, IFN-γ and lower IL-10 response in the C57BL/6 strain compared to BALB/c and C3H. Hence, early increases in the levels of the pro-inflammatory cytokines were associated with earlier eradication of infection. Because of the higher Th1 response in C57BL/6 mice, they sustained a shorter course of infection and less oviduct pathology than did the C3H mice during the first week of infection.

Additional cytokine studies have shown that mice depleted in interleukin-12 (IL-12) or IFN-γ and/or the IFN-γ receptor have an inability to clear primary chlamydia infection (Cotter *et al.* 1997; Johansson *et al.* 1997, and Perry *et al.* 1997). Interferon gamma has been shown to play a vital role in the clearance of chlamydia. The two IFN-γ-inducible host cell functions that have received the majority of attention in studies of chlamydia immunity are the induction of inducible nitric oxide synthase (iNOS) and of tryptophan-decyclizing enzyme indoleamine 2, 3-dioxygenase (Morrison and Caldwell, 2002). To determine the role of nitric oxide (NO), Igietseme *et al.* (1998) used iNOS knockout mice and analyzed the effect of NO on *in vivo* and *in vitro* growth of Chlamydia and reported that the iNOS pathway was not required for elimination of chlamydia from epithelial cells lining the female genital tract, however NO may control the dissemination of *C. trachomatis* by infected macrophages. Indoleamine 2, 3-di-oxygenase (IDO) is an enzyme that catalyzes the decyclization of L-tryptophan into N-formylkynurenine. L-tryptophan is an essential amino acid for chlamydia growth (Byrne, et al. 1986). Therefore, depletion of L-tryptophan inhibits growth.

Chlamydia infected mice that were IL-6 and TNF-α receptor deficient and depleted of TNF-α had minimal or no measurable effect in the ability to resolve primary infection or resist against secondary challenge (Darville *et al.* 2000, Perry *et al.* 1998). These results demonstrate the importance of Th1 immunity in resistance to chlamydial infection. Moreover, in a study by R. Johnson (2004), it was concluded that a cloned cell line infected by *C. muridarum* secreted TNF- α, a major activator of neutrophils within infected tissues. Tumor Necrosis Factor-α, IL-1α, and GM-CSF made by epithelial cells would activate macrophages and trigger dendritic cells maturation. IL-6 is an important cytokine in cytotoxic T-cell differentiation (Johnson 2004) (Figure 2.) In contrast, studies investigating *Neisseria gonorrhea,* a bacterium associated with another well known STD, gonorrhea, suggested that IL-8, IL-6, and possible tumor necrosis factor alpha (TNF-α) were produced at the local site of infection.

These cytokine responses appear to be directed by the differential expression of chemokine receptors on Th1- and Th2- cell subsets (Belay 2002). The identification of several new chemokines has revealed that chemokines also play an important role coordinating the movement of T cells, B cells and dendritic cells necessary to generate an immune response. Chemokine messages are decoded by specific receptors that initiate signal transduction events leading to a multitude of cellular responses, leukocyte chemotaxis and adhesion in particular. Receptor expression in different leukocyte subsets dictates to a large extent the spectrum of action of chemokines, and differentiation or cellular activation modulates receptor expression (Baggiolini *et al.* 1997). Bonecchi *et al.* (1998) reported that human Th1s and Th2s differentially express chemokine receptors and, accordingly, differentially migrate in response to different

chemokines. In addition, this investigator also observed cells bearing the chemokine receptors chemokine receptor 5 (CCR5) and chemokine receptor 3 CXCR3, which are known to be expressed on Th1 and dendritic cells.

Interestingly, Belay *et al.* (2002) showed that CCR5 are expressed on the T cells of mice infected with genital chlamydial infection. Chemokine receptor-5 plays an important role in various inflammatory diseases, infections and atherosclerosis. Various studies have indicated that CCR5-deficient mice were significantly more susceptible to infections with the parasites *Listeria monocytogenes, Cryptococcus neoformans* and *Toxoplasma gondii* (Khan *et al.* 2006). Moreover, they displayed an increased mortality in influenza A virus infection. Studies have also shown that CCR5 and CCR2 knockout mice appear to have a partial defect in macrophage function and show a decreased Th1 immune response, as measured by a reduced production of Th1 cytokines, such as IFN-γ (Yang *et al.* 1998; Patton and Rank 1992) and IL-12 (Mack 2001). Mack and colleagues successfully developed mouse monoclonal antibodies (mAbs) against the murine chemokine receptor CCR5 and found that the absence of this receptor decreased the inflammation that is usually observed in inflammatory diseases such as nephritis and arthritis. R. Gallos (2000) laboratory showed the first clue that chemokine-related events were important in HIV pathogenesis. Their investigation showed that high levels of chemokines could inhibit HIV replication in vitro (McNicholl 2003). A cohort of highly exposed, HIV-negative men had high circulating levels of several chemokines, such as RANTES, MIP-1α, and MIP-1β. This finding led others to conduct studies, which ultimately concluded that CCR5 plays a pivotal role by acting as a co-receptor with CD4 for the entry of HIV (McNicholl 2003).

Chemokine receptor-5 has also been shown to be a critical receptor in guiding natural killer cells trafficking in host defense. With the influx of NK cells, tissues from CCR5-deficient (CCR5-/-) mice were more susceptible to infection with *Toxoplasma gondii* infection (Khan *et al.* 2006). In studying other bacterial infections, it has been shown by Peters *et al.* (2001) that CCR2 (chemokine receptor 2), another Th1 receptor, serves an early and essential role in resistance to *Mycobacterium tuberculosis.*

As a crucial receptor involved in T cell activation and function, a deficiency of CCR5 is associated with a suppression of T cell induction and leukocyte migration under certain infectious and non-infectious conditions, suggesting that it plays a role in both infection-related immune and inflammatory processes. The effect of a targeted suppression of the critical specific T cell response on both immune-mediated microbial clearance and the development of complications of chlamydial infection is largely unknown.

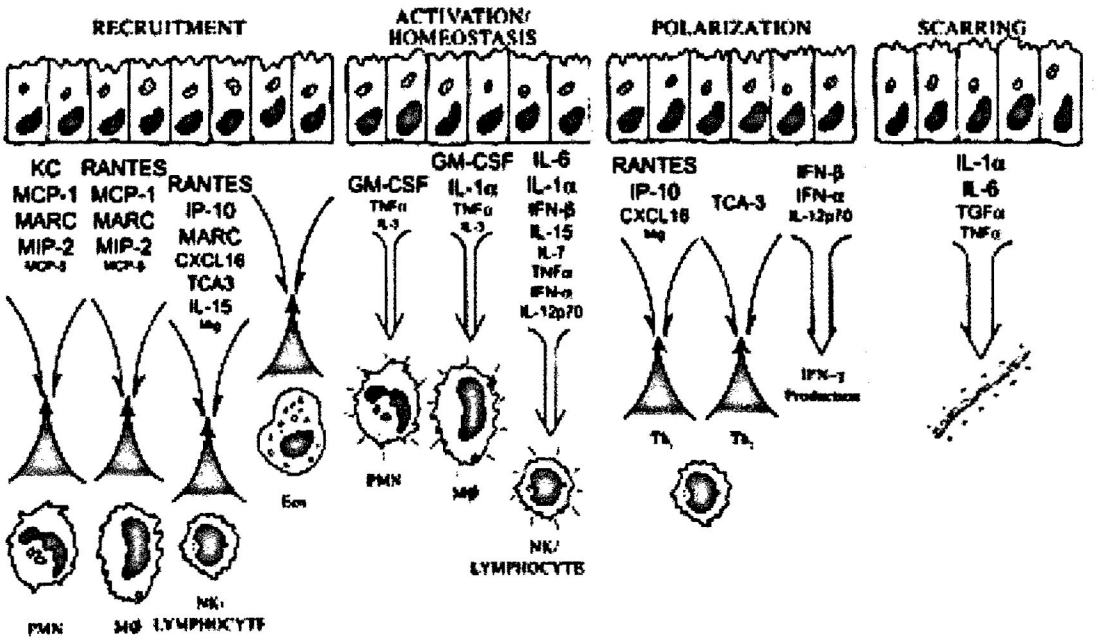


Figure 2. Summary of Bm1.11 oviduct epithelial cell cytokine responses to infection by *C. muridarum*. Relative expression levels are reflected by type sizes within the figure, with 4+ represented by the largest type size [e.g., MARC (CCL7)], 3+ represented by the second-largest type size (e.g., CXCL16), 2+ represented by the second-smallest type size (e.g., TNF-α), and 1+ indicated by the smallest type size (e.g., IL-3). PMN, polymorphonuclear cell; Eos, eosinophil (Johnson *et al.* 2004)

Hormonal Environment. Estrogens are aromatized steroid hormones synthesized from a cholesterol backbone and produced predominately in the ovary, although some aromatization may occur in adipose tissue. Estrogen production increases gradually throughout female development. Estrogens may occur in many forms: 17-β estradiol is the most common circulating form. Precursors to estrogen in the steroid biosynthetic pathway include progesterone, dehydroepiandrosterone (DHEA), and testosterone (McMurray 2001)

Estrogen modulates lymphoid cell growth and differentiation, proliferation, antigen presentation, cytokine production, antibody production, cell survival, and apoptosis (McMurray 2001). Although few studies have dissected direct hormonal effects on cytokine profiles in human lymphohematopoietic cell subsets, $CD4^+$ T cell clones from multiple sclerosis (MS) patients have shown cytokine-specific dose-dependent response to estrogen (Cohen *et al.* 1983). In addition, progesterone facilitates production of Th2 cytokines in normal T cell clones (Piccinni *et al.* 1995). Vassiliaudou *et al.* (1999) determined whether progesterone affected mechanisms underlying the sexual transmission of HIV-1 and observed that hormonal treatment had no effect on expression of CCR5 and CXCR4 by nonactivated T cells and macrophages, but significantly inhibited IL-2-induced up-regulation of CCR5 and CXCR4 on activated T

cells. In addition, this group also concluded that progesterone inhibited both mitogen-induced proliferation and chemokine secretion (MIP-1α, MIP-1β, and RANTES) by CD8+ T lymphocytes.

Epidemiologic evidence suggests that infection with *C. trachomatis* is enhanced by oral contraceptives (Washington *et al.* 1985). This link between hormonal contraception and cervical infections is biologically plausible because estrogen and progesterone can enhance or suppress the growth and persistence of vaginal flora. In a quantitative review by Cottingham and Hunter (1992), it was observed that women who chose Depo-Provera as a contraceptive experienced a three-fold increase in the risk of acquiring two of the most commonly spread bacterial STDs; chlamydial and gonorrhea. Although these findings were interesting, the study did not look into the behavioral actions that could have attributed to the increased risk.

In vitro studies have shown that treatment of HeLa 229 cultures with 17-β-estradiol, and a synthetic analog diethylstilbesterol prior to infection with *C. trachomatis* serovar K or serovar L1 led to a 50 to 60% enhancement of chlamydial inclusion formation (Bose and Goswami 1986). This enhancement was reversed when cells were treated with an anti-estrogen tamoxifen.

Sex hormones regulate immune responses in the female reproductive tract. The estrous cycle and treatment with sex hormones such as estradiol and progesterone, have been demonstrated to influence mucosal immune elicitation and function in the genital mucosal surface, cytokine expression and recruitment or trafficking of leukocytes into the reproductive tract (Jungi *et al.* 1997; Prabhala *et al.* 1995). Kaushic *et al.* (1998) used a rat model to study the effects of sex hormones on *C. trachomatis*. It was concluded that

enhanced lymphocyte proliferation was observed in response to mitogenic and MOMP stimulation in the reproductive tract-draining lymph node (LN) from estrous and diestrous animals. In addition, it has also been noted that pre-treating guinea pigs with 17 β-estradiol prior to infection enhanced the course of infection of guinea pig GPIC (Rank *et al.* 1982). These findings are comparable to others stating that estrogen's effect on vaginal candidiasis and *N. gonorrhea.* The incidence of vaginal candidiasis increases with the use of oral contraceptives (Washington 1985). Mice are more susceptible to genital infection with *N. gonorrhea* at proestrus, when estrogen levels are higher (Kita *et al.* 1981).

Although, there is supporting evidence that sex hormones have an effect on bacterial infection, the cellular, and molecular and biochemical mechanisms underlying this influence on genital infections by specific microbial pathogens have not been clearly identified (Sonnex *et al.* 1998).

The scope of this work was to investigate estrogen's effect on the induction of protective immunity using *in-vitro* and *in-vivo* models.

CHAPTER 3

MATERIALS AND METHODS

Materials

Animals

Inbred, chemokine receptor-5 KO ($CCR5^{-/-}$), and wild type female mice, 5-8 week old, were purchased for these studies.

Mouse Strain	Knockout gene	Designation	Source
C57BL	**CCR5-/-**	**CCR5 KO**	**Jackson Laboratory** (Bar Harbor, ME)

All animals were housed in the animal facility of the Atlanta University Center (AUC), located at Morehouse School of Medicine (Atlanta, GA). Animals were fed food and water *ad libitum*, and maintained in laminar flow racks under pathogen-free conditions (Trexler germ-free isolators) of 12-hr light and 12-hr darkness. Supervision of animal care was under a licensed veterinarian and a qualified manager, Mr. Clarence Wilkes (Morehouse School of Medicine, Atlanta, GA).

Antigen

Stocks of the *Chlamydia trachomatis* agent of mouse pneumonitis (*C. muridarum*/MoPn) were generously provided by Drs. Joseph Igietseme and Carolyn Black at the Centers for Disease Control and Prevention (CDC, Atlanta, GA).

Cell Lines

McCoy This is a murine fibroblast cell line that has been used to propagate laboratory strains of all recognized *C. trachomatis* serotypes (Hodinka *et al.* 1998) designated American Tissue Cells Culture (ATCC) CRL-1696. Cells were purchased from ATCC (Manassas, VA).

HeLa This is an epithelial-like cell line derived from human cervical carcinoma tissue, designated at ATCC CCL-2, and was purchased from ATCC. HeLa cells have been found to efficiently support the growth of MoPn in culture.

Tissue Culture Reagents and Materials

Cycloheximide Medium (CX). Sterile, filtered complete CX medium contained 10% Fetal Bovine Serum (FBS) (heat inactivated at 56 °C for 30 minutes) (Atlanta Biological, Norcross, GA), 3 mg/ml of glucose (Atlanta Biological, Norcross, GA), 100 µg/ml of vancomycin (Dr. Joseph Igietseme, MSM, Atlanta, GA), 100 µg/ml of gentamycin (Atlanta Biological, Norcross, GA), 1.25 µg/ml of fungizone (Atlanta Biological, Norcross, GA), and 0.5 µg/ml of cycloheximide (Sigma, St. Louis, MO) in Dulbecco's modified eagle medium (DMEM, Atlanta Biological, Norcross, GA). Media was stored at 4°C until use.

Complete Dulbecco's Modified Eagle Medium (DMEM). Sterile, filtered complete DMEM contained 100mM sodium pyruvate (Atlanta Biological, Norcross, GA), 10 mM non-essential amino acids (Atlanta Biological, Norcross, GA), 1M HEPES (Atlanta Biological, Norcross, GA), 100X insulin/transferring/selenium (ITS, Dr. Carolyn Black, CDC, Atlanta, GA), 100 mM L-glutamine (Atlanta Biological, Norcross, GA), 50 mg/ml gentamycin (Atlanta Biological, Norcross, GA), 240 µg/ml

fungizone/amphotericin B (Atlanta Biological, Norcross, GA) and 10% fetal bovine serum (heat inactivated at 56 °C for 30 minutes, Atlanta Biological, Norcross, GA) in Dulbecco's modified eagle medium (DMEM, Atlanta Biological, Norcross, GA). Media was stored at 4 °C until use.

Transport Medium. Sterile, filtered transport medium contained 50 mg/ml gentamycine (Atlanta Biological, Norcross, GA), 250 µg/ml fungzone/amphotericin B (Atlanta Biological, Norcross, GA), 10% FBS (heat inactivated at 56 °C for 30 minutes; Atlanta Biological, Norcross, GA), 100 µg/ml vancomycin (Dr. Joseph Igietseme, MSM, Atlanta, GA) and 2-SP buffer (prepared in the laboratory-see methods). Media was stored at 4 °C until use.

Roswell Park Memorial Institue (RPMI) Complete Medium. Sterile, filtered RPMI complete medium contained RPMI-1640 (Atlanta Biological, Norcross, GA) with 10% FBS (Atlanta Biological, Norcross, GA), 100 mM sodium pyruvate (Atlanta Biological, Norcross, GA), HEPES (Atlanta Biological, Norcross, GA), 200 mM L-glutamine (Atlanta Biological, Norcross, GA), 1 M Penicillin-Streptomycin (Pen-Strep, Atlanta Biological, Norcross, GA), and 1:200 dilution of 55 mM 2-mercaptoethanol (2-ME, Gibco BRL, Life Technologies, Gaithersburg, MD) diluted in RPMI-1640. Media was stored 4 °C until use.

RPMI Complete Medium without Penicillan-Streptomycin (Pen-Strep). Sterile, filtered RPMI complete medium contained RPMI-1640 (Atlanta Biological, Norcross, GA) with 10% FBS (Atlanta Biological, Norcross, GA), 100 mM sodium pyruvate (Atlanta Biological, Norcross, GA), 10 mM non-essential amino acids (Atlanta Biological, Norcross, GA) 1M HEPES (Atlanta Biological, Norcross, GA), 200 mM L-

glutamine (Atlanta Biological, Norcross, GA), 50 mg/ml gentamycin (Atlanta Biological, Norcross, GA) and 1:200 dilution of 55 mM 2-mercaptoethanol (2-ME, Gibco BRL, Life Technologies, Gaithersburg, MD) diluted in RPMI-1640. Media was stored at 4 °C until use.

Tissue Culture Flasks and Plates. 75 cm^2 and 150 cm^2 flasks and 60 x 15 mM plastic Petri dishes were purchased from Corning Costar Corporation (Cambridge, MA). Tissue culture plates (i.e. 6-, 24-, 96-well flat and round-bottom plates) were purchased from Corning (Corning, NY).

Other Reagents and Materials

Phosphate-Buffered Saline (PBS). Sterile, filtered 1X PBS (pH. 7.4) contained 8 g of NaCL (Gibco, BRL, Life Technologies, Gaithersburg, MD), 0.2 g of KCL (Fisher Scientific, Pittsburgh, PA), 1.15 g of Na_2HPO_4 (Fisher Scientific, Pittsburgh, PA), and 0.2 g of KH_2PO_4 (Fisher Scientific, Pittsburgh, PA) in 1 liter of dH_2O. PBS was stored at 4 °C until use.

Lysing Buffer. Tris-Ammonium Chloride (NH_4Cl) (pH. 7.2) contained 7.5 g NH_4Cl (Fisher Scientific, Pittsburgh, PA) and 2.05 g of Tris (Fisher Scientific, Pittsburgh, PA) in 1 liter of dH2O. The lysing buffer was used to remove red blood cells from leukocyte preparations according to a previously described procedure (Moore 2002). The pH was adjusted to 7.2 and the solution was stored at 4 °C until use.

Trypan Blue. Trypan Blue at a concentration of 0.4% prepared in 0.85% saline solution (Gibco BRL, Life Technologies, Gaithersburg, MD) was used to detect cell viability.

Enzyme-Linked Immunosorbent Assay (ELISA) Reagents. ELISA kits for quantitating the amounts of murine cytokines in biological and culture fluids were purchased from BioSource International (Camarillo, CA). All necessary assay components (i.e. 1st and 2nd antibodies, blocking reagents and washing buffer) were supplied with each individual kit and used according to the manufacturer's protocol.

Instrumentation

Fluorescent Microscope. Zeiss (Zeiss, Atlanta, GA) fluorescent microscope was used for the immunofluorescence visualization and enumeration of chlamydial inclusions.

Hemacytometer. Bright line hemacytometer (Hausser Scientific, Horsham, PA) 0.1 mm deep, was used to enumerate viable cells using the trypan blue exclusion method.

Incubator. NuAire™ IR Autoflow CO_2 Water-Jacketed Incubator (Med-Rep Inc., Newnan, GA) was used to incubate cell and tissue cultures at 37°C and 5% CO_2.

Gamma-Irradiator. Gammacell 1000 EliteTM gamma-irradiator (Nordion International, Inc., Kanata, Canada) was graciously made available through the laboratory of Dr. Godwin Ananaba (Clark Atlanta University, Atlanta, GA) to irradiate murine spleen cells.

Spectrophotometer. Gene Quant II ™ (Amersham Pharmacia Biotech, Piscataway, NJ) was used to quantify RNA absorbance levels in cell culture samples.

Centrifugation. Marathon 12 KBR Centrifuge (Fisher Scientific, Pittsburgh, PA) and Eppendorf Centrifuge 5402 (Brinkman Scientific, Westbury, NY) were used throughout experimentation.

Micro-Titer Plate Reader. Molecular Devices SpectraMax 250^{TM} (Sunnyvale, CA) was used to quantify murine cytokine levels elaborated by biological and culture fluids during ELISA experiments.

Methods

Animal Development

Chemokine Receptor-5 Knockout Mice (CCR5KO). CCR5KO mice (C57BL/6 background) were developed by gene targeting inactivation in the laboratory. CCR5 is a seven transmembrane-spanning G-protein-coupled-receptor. It is expressed in lymphoid organs such as thymus and spleen and on monocytes, macrophages, T-cells and B-cells. Zhou *et. al.* have generated mice lacking expression of CCR5. These mice develop normally in a pathogen-free environment, but show reduced efficiency in clearance of Listeria infection.

Chlamydial Stock and Antigen Preparation

Stocks of the *Chlamydia trachomatis* of mouse pneumonitis (C. muridarum/MoPn) were prepared by propagating elementary bodies (EBs) in McCoy cells (Ramsey *et al.* 1988). Briefly, MoPn was cultured in McCoy cells in DMEM containing 10% FBS (see materials). MoPn grown in McCoy cells was stored at -80°C and thawed just before use. The cell suspension was sonicated and centrifuged at 192 x g for 10 minutes at 4 °C. The pellet was then suspended in sucrose-potassium glutamate buffer (pH 7.2).

Stocks were titrated by infecting McCoy cells with varying dilutions of EBs. To summarize, a 96-well flat bottom tissue culture plate (Corning, Corning New York) containing 200,000 McCoy cells/well was incubated at 37 °C (5%/CO_2) overnight to

produce a monolayer. The following day, serial dilutions (i.e. 10^{-4}, 10^{-5}, and 10^{-6}) of *Chlamydia* stock in CX media were added to each well. Wells containing CX media alone were used as controls. Plates were centrifuged at 3000 rpm for one hour at 34 °C. After centrifugation, plates were incubated for 2 hours at 37 °C (5% CO_2). Afterwards, medium was aspirated from each well using disposable borosilicate glass pasteur pipet (Fisher Scientific, Pittsburgh, PA) and fresh, warm CX medium was added to each well. Plates were incubated further at 37 °C, 5% CO_2 for 32 hours. Following incubation, plates were fixed with methanol (histological grade, Fisher Scientific, Pittsburgh, PA) and stained with 1:1 dilution in sterile PBS of FITC-conjugated anti-chlamydial antibodies (PathoDx Chlamydia Culture Confirmation Kit, Diagnostic Products Corporation, Los Angeles, CA). Inclusions were visualized and enumerated by immunofluorescence (Ramsey *et al.* 1988). The infectious titer was expressed as inclusion-forming units per milliliter (IFU/ml). Chlamydial antigen was prepared by growing MoPn in HeLa cells and purifying EBs over a renograffin gradient, followed by inactivation under ultraviolet light for 3 hours.

Intravaginal Inoculation of Mice

Depo-Provera Treatment. Seven days prior to intravaginal infection, mice were given a single subcutaneous injection of 0.1 ml of a 1:16 dilution (25 mg/ml) of Depo-Provera (6-alpha-methyl-17-alphahydroxy progesterone acetate, Sigma, St. Louis, MO) in phosphate buffered saline (PBS). Depo-Provera is a progesterone derivative that stimulates the negative feedback center of the hypothalamus and inhibits the release of gonadotropin-releasing hormone (GnRH). GnRH acts on the anterior pituitary to induce follicle-stimulating hormone (FSH) and lutenizing hormone (LH) secretion. FSH induces

growth of follicles and follicular estrogen production. Thus, Depo-Provera drives mice to anestrus enabling the estrous cycle to be halted long enough for productive chlamydial genital infection to be established. The applied dosage had been found to last approximately 20 days (Tuffrey *et. al.* 1986; 1984).

A solution of Depo-Provera was prepared by adding 250 mg of Depo-Provera to 10 ml of filter-sterilized PBS in a 15 ml disposable polypropylene Fisherbrand tube with a plug seal cap (Fisher Scientific, Pittsburgh, PA). The solution was placed in a 37 °C water bath for 10 minutes, followed by syringe filtering with a 3 cc syringe (Becton Dickinson & Co., Franklin Lakes, NJ) with a Precision Glide 30 G1/2 needle (Becton Dickinson & Co., Franklin Lakes, NJ). Depo-Provera was administered through subcutaneous injection in the vaginal region of the mouse.

Anesthetizing Mice for Intravaginal Inoculation. On the day of infection, mice were anesthetized with 0.1 ml of a 1:10 dilution of 50 mg/ml Nembutal (Sodium Phenobarbital) provided by Dr. Joseph Igietseme, (Morehouse School of Medicine, Atlanta, GA) in sterile filtered PBS. Nembutal was syringe filtered before use. A 1 ml latex free syringe (Becton Dickinson & Co., Franklin Lakes, NJ) with a Precision Glide 30G ½ needle (Becton Dickinson & Co., Franklin Lakes, NJ), was used to administer the intraperitoneal injection. Mice were completely anesthetized before inoculation occurred.

Infection Protocol. Mice were individually infected with 30 µl of PBS containing 107 IFU/ml of the *C. trachomatis* agent of mouse pneumonitis (*Chlamydia muridarum* or MoPn). A Clay Adams P200 pipetman (Becton Dickinson & Co., Franklin Lakes NJ and Clay Adams siliconized micro-selectapette pipette tips (Becton Dickinson & Co.,

Franklin Lakes, NJ) were used to for intravaginal inoculation of mice. Mice were then placed on their backs within their respective cages until anesthesia wore off. The mice were checked 3 hrs after infection for possible adverse reaction(s).

The course of infection was monitored by cervical-vaginal swabbing, every three days for six weeks, a time period that has been found to span the course of *C. trachomatis* genital infection in mice (Patton and Rank 1992; Rank 1999; Khamesipour *et al.* 1994; Igietseme and Rank 1991). Mice were swabbed for isolation of chlamydiae from the vaginal vault and sacrificed to obtain the spleen and genital tract. Experiments were repeated to give 10 to 12 animals per experimental group.

Isolation of Chlamydia

Cervical-vaginal Swabbing of Murine Genital Tract for Isolation of Chlamdyia. *Chlamydiae* were isolated from mice by cervical-vaginal swabbing using individually wrapped sterile calcium alginate tipped aluminum applicator swabs (Fisher Scientific, Pittsburgh, PA). Swabs were maintained in sterile, filtered transport medium (see materials). One milliliter of transport medium was added to labeled, autoclaved 16 x 10 mm borosilicate screw-top glass tubes (Kimble, Fisher Scientific, Pittsburgh, PA). Sterile forceps were used to add two autoclaved 6 mm glass beads (Fisher Scientific, Pittsburgh, PA) to each tube. Tubes were stored at 4 °C until use. Tubes were placed on ice while individual mice were obtained from designated cages and the genital region was swabbed vigorously for 30 seconds. Swabs were stored at -80 °C until processed.

Tissue Culture Preparation. Chlamydia was isolated from swabs into tissue culture. McCoy or HeLa cells were grown and maintained in complete DMEM. Cells in a confluent monolayer were trypsinized using 0.25% trypsin to detach them from the tissue

culture flasks and centrifuged at 1230 rpm for 30 minutes at 4 °C in 50 ml Fisherbrand tubes (Fisher Scientific, Pittsburgh, PA). Following centrifugation, cells were re-suspended in complete DMEM and counted using a bright-line hemacytometer (Hausser Scientific, Horsham, PA) (see materials). Cells (10^5) were plated in DMEM in tissue culture-treated polystyrene sterile non-pyrogenic 96-well flat bottom plates.

Isolation from Genital Swabs into Tissue Culture. Following the preparation of a confluent cell monolayer, frozen (-80 °C) genital swabs were placed in 1 ml of warm cyclohexamide (CX) complete medium and vortexed vigorously for 2 minutes to allow detachment of chlamydiae from the swabs. Two hundred microliters of the CX suspension containing chlamydiae was added to the cell monolayer in triplicates.

Tissue culture plates containing the genital swab supernatants were centrifuged at 3000 rpm for 1 hour at 34 °C. Following centrifugation, plates were incubated at 37 °C (5% CO_2) for 2 hours. CX medium was then aspirated from the plates and 200 µl of fresh CX medium was added. Plates were incubated for an additional 32 hours at 37 °C (5% CO_2). Once the incubation period had expired, plates were removed from the incubator and the CX media was aspirated from the plates. Sterile 100% methanol (200 µl) was added to each well and plates were placed in either 4 °C for 5 hours or at room temperature for 2 hours to allow fixation to occur. Afterwards, methanol was removed from plates by inversion, followed by washing with sterile PBS.

Thirty microliters of fluorescein isothiocynate (FITC)-labeled, genus-specific purified murine monoclonal antibodies (1:1 dilution of PBS, Chlamydia culture confirmation kit, PathoDx™, Diagnostic Products Corporation, Los Angeles, CA) were added to each well of the methanol-fixed tissue culture plates. Plates were allowed to

stand at room temperature in the dark for 1 hour. To remove the non-binding stain, plates were washed twice with sterile PBS. Following the wash, plates were inverted into absorbent paper, covered with aluminum foil and left overnight to allow for removal of PBS. The following day, one drop of glycerol (90% in PBS) was added to each well. Plates could then be viewed and chlamydial inclusions would be enumerated using a fluorescent microscope (Ramsey *et al.* 1986). For each well, a minimum of 10 fields was counted, and the average number of inclusions was recorded.

Cell Population Preparations

Preparation of Splenic T lymphocytes. Spleen cells were enriched for T cells by the nylon wool adherence method (Igietseme *et al.* 1993). Naive or immunized mice were sacrificed by cervical dislocation and the entire body cleansed with 70% ethanol. The spleens were aseptically removed and placed into RPMI 1640 medium with 5% FBS and 1% HEPES. Isolated spleens were teased with sterile forceps to remove large tissue clumps. Cell suspensions were treated with lysing buffer for 5 minutes at 37 °C. Cells were then washed three times by centrifugation at 1300 rpm for 25 minutes at 25 °C. Following the final wash, cells were re-suspended in 3 ml of RPMI complete medium (without pen-strep) and run twice through a nylon wool column using warm RPMI complete medium.

Nylon wool columns were pre-treated the day of use by calibration with RPMI with 5% FBS and 1% HEPES complete medium. Columns were then incubated for 45 minutes at 37 °C. Splenic cells suspensions were run through a 20 G1/2 needle placed into a stopcock on a 20 cc syringe. Following, nylon wool extraction, spenic T cells were centrifuged at 1300 rpm for 40 minutes at 25 °C. Cells were re-suspended in 5 ml RPMI

complete medium (no pen-strep), enumerated using a hemacytometer and ready for experimental use. Purified splenic cells contained at least 90% $CD4^+$ cells as was determined by FACS analysis (Moore et al. 2002)

Preparation of Splenic Antigen Presenting Cells. Naïve mice were sacrificed by cervical dislocation and the entire body cleansed with 70% ethanol. The spleens were removed surgically using aseptic techniques into RPMI 1640 medium with 5% FBS and 1% HEPES. Isolated spleens were teased with sterile forceps to remove large tissue clumps. Cell suspensions were treated with lysing buffer for 5 minutes at 37 °C. Cells were washed three times by centrifugation at 1300 rpm for 25 minutes at 25 °C. Following the last wash, cells were re-suspended in 3 mls of RPMI complete medium (without pen-strep) and gamma-irradiated (Gammacell 1000 ElilteTM gamma irradiator, see materials). Following gamma-irradiation, splenic antigen presenting cells were centrifuged at 1300 rpm for 40 minutes at 25 °C. Cells were re-suspended in 5 ml of RPMI complete medium (no pen-strep) and enumerated using a hemacytometer.

Isolation, Reverse Transcription, Real-Time Polymerase Chain Reaction (RT-PCR) in Estrogen Treated and Untreated McCoy cells.

Ribonucleic Acid (RNA) Isolation. Total RNA was extracted from Mopn-infected McCoy cells that were pretreated with 17 β-estradiol using RNeasy manufacturer's protocol (Qiagen, CA). Briefly, Cells were lyzed using Buffer RLT (contains guanidine thiocyanate). Lysate was pipetted directly into a QIAshredder spin column (supplied by the manufacturer) and centrifuged for 2 minutes. One volume of 70% ethanol was added to the homogenized lysate, and mixed well by pipetting. The sample was transferred to an RNeasy spin column (supplied by manufacturer) and placed in 2 ml collection tube.

The tubes were centrifuged for 15 s at ≥ 10,000 rpm. Buffer RW1 was added to RNeasy spin column (700 µl) and centrifuged for 15 s at ≥ 10,000 rpm to wash the spin column membrane. Buffer RPE was added to the spin column and centrifuged for 15 s to wash the spin column. The RNeasy spin column was placed in a new 1.5 column tube. RNase-free water was added directly to the spin column membrane and column was centrifuged for 1 min ≥ 10,000 rpm to elute RNA. Total RNA isolated was quantified by measuring absorbance at 260 nm and 280 nm. The RNA samples were aliquoted into 5 µg aliquots and stored at -80 °C.

Reverse Transcription of Purified RNA to Complementary Deoxynucleic Acid (cDNA). Isolated RNA was used for first strand synthesis using JumpStart AccuTaq™ LA DNA Polymerase Mix (Sigma, St. Louis MO). Briefly, one microliter of random nanomers (2.5 µM) was added to a tube containing 5 µg RNA and deoxynucleotide mix (500 uM dNTP) was added to 5 ul of PCR reagent water to give 10 µl total volume. The mixture was briefly centrifuged and placed in a 70 °C water bath for 10 minutes. Following incubation, the tube was placed on ice. The following reagents were added: PCR reagent water (6µl), 10X buffer for AMV-RT (2µl), RNase inhibitor (1µl), enhanced avian RT (1µl) and reaction tubes incubated at 25 °C for 15 minutes. Reaction tubes were placed at 50 °C for 50 minutes. The first strand of cDNA was placed at 20 °C for subsequent PCR amplication.

PCR Amplification of Target cDNA. To amplify mouse cDNA, custom-made primers were used (Table 1, Invitrogen, California). PCR amplification was carried out in a Perkin-Elmer DNA Gene AMP 2400 thermocycler. Each tube contained a total volume of 50 ul: 5 µl of cDNA from RT reaction, 5 µl 10X AccuTaq buffer (1X), 1 µl

dNTP (200uM), 2 µl JumpStart AccuTaq LA DNA polymerase mix (0.05 units/ µl) and 0.4 µM of primer (forward and reverse). Sterile water was used to adjust the volume in thin-walled reaction tubes. Conditions for DNA amplifications were set as follows: heating at 94 °C for 3 minutes followed by 35 cycles of DNA denaturing for 94 °C for 1 minute, annealing at 58 °C for 1 minute, an extension at 72 °C for 1 minute with a final extension step at 72 °C for 10 minutes. Equivalent amounts of cDNA template were used in reactions with primers specific for β-actin, as well as negative controls containing no cDNA.

Ten microliters of each PCR product was combined with 2 µl of loading buffer (5X) and 2 µl of sterile water and electrophoresed on a 1% agarose (Sigma, CA) gel containing 0.5 µg/ml ethidium bromide (Sigma, St. Louis, MO) in 1X Tris-Acetate/EDTA buffer (TAE, Gibco BRL, Grand Island, NY).

Quantitative PCR SYBR Green ReadyMix for High Throughput Quantitative PCR was used to measure mRNA expression. The following was added to a thin-walled PCR tube: 25 µl SYBR Green ReadyMix (20 mM Tris-Hydrochloric Acid), pH. 8.3, 25 °C, 100 mM Postassium Chloride (KCL), 7 mM Magnesium Chloride ($MgCL_2$), 0.4 mM each dNTP, stabilizers, 0.05 unit/ul Taq DNA Polymerase, Jumpstart Taq antibody, 2X internal reference dye and SYBR Green I), 1 µl primers, 0.2 µM (forward and reverse), 2 µl cDNA and brought up to 25 µl with PCR reagent water. Reaction tube was gently vortexed and centrifuged. Conditions for DNA amplification were set as follows: initial denaturation at 95 °C for 2 minutes followed by 35 cycles of DNA denaturation at 95 °C for 1 minute, annealing at 55 °C for 30 seconds, an extension at 72 °C for 1 minute with a final extension step at 72 °C for 2 minutes.

Assay Design

The Effects of Estrogen on the Expression of Chemokines and Adhesion Molecules during Chlamydia Infection of Epithelial Cells

Assessment of Estrogen's Effect on *Chlamydial* growth in Mouse Epithelial Cells. McCoy cells were grown to 70% confluency in complete DMEM. Once cells reached confluency, regular DMEM was removed and replaced with phenol-red free DMEM containing 10% charcoal-dextran treated FBS. After 24 hours, cells were treated with various molar concentrations of 17 β-estradiol (10^{-8}, 10^{-9}, 10^{-11}). Estrogen treated cells were incubated at 37 °C with 5% CO_2 for 24 hours. Post incubation, cells were infected with MoPn. Following 32 hours, media was removed and cells were assessed for chlamydia infection using immunofluorescence (see methods).

Assessment of Estrogen's Effect on Chemokine Production in Mouse Epithelial Cells during Chlamydia Infection. McCoy cells were grown to 70% confluency in complete DMEM. Once cells reached confluency, DMEM was removed and replaced with phenol-red free DMEM containing 10% charcoal-dextran treated FBS. After 24 hours, cells were treated with various molar concentrations of 17β-estradiol (10^{-8}, 10^{-9}, 10^{-11}). Estrogen treated cells incubated at 37 °C with 5% CO_2 for 24 hours. Post incubation, cells were infected with MoPn. Following 32 hours, supernatants were collected from cells and assessed for chemokine (RANTES, MIP-1α, MIP-1β, IP-10, MCP-1) profiles using ELISA (see methods). Experiments were repeated three times.

To determine mRNA expression RNA was collected from treated and infected cells and RT-PCR was performed according to methods described above.

Determination of the Effects of Estrogen on Chlamydia infectivity and Chemokine/Cytokine Production in a Mouse Model

In Vitro Infectivity Assay to Quantitate Chlamydial Shedding in Vaginal Washes

Estrogen (17 β-estradiol: Sigma Chemical Co, St. Louis MO, dissolved in absolute ethanol at a concentration of 0.01 M and diluted in saline, 10^{-8} M) was administered to mice intramuscularly for 7 consecutive days. At day 7 mice were infected intra-vaginally with 10^7 IFU of MoPn per mouse. Control mice received saline only. The course of infection was followed up to five weeks. *Chlamydiae* were isolated from the swabs in tissue culture and enumerated by immunofluorescence. The results were repeated to include 10-12 animals per group.

Chlamydia-induced cell activation, cytokine and chemokine secretion by leukocytes. At various times post infection (7, 14, 21 and 38 days) mice were sacrificed by cervical dislocation. The spleen and genital tracts were harvested and placed in RPMI (5% FBS, 1% HEPES) and stored on ice until further use. The genital tracts were homogenized, and T cells isolated by the nylon wool method. The homogenate containing lymphocytes was stimulated with UV inactivated antigen and APCs for approximately 5 days. After 5 days the supernatants were collected and analyzed for chemokines and cytokines (RANTES, MIP-1α, MIP-1β, IP-10, KC, IL-4, IL-10, TNF-α, IL-1β, IL-6 and IL-8) using ELISA as described earlier. The Th1 response was measured by isolating the T cells from the spleen harvested from the estrogen treated and chlamydia infected animals using the nylon wood method. The isolated T cells were stimulated with APCs from infected WT mice (2×10^5 cells/well) and UV-inactivated antigen (10 μg/ml). After a five day incubation at 37 °C (5% CO_2), the supernatants were

assayed for IFN-γ by quantitative ELISA. The concentration of the cytokine and chemokines in each was obtained by extrapolation from a standard calibration curve generated simultaneously. Data were calculated as the mean values (± standard deviation) of triplicate cultures for each experiment. The results were derived from at least 3 independent experiments.

Evaluation of Estrogen on fertility during *chlamydia* infection. Estrogen (17 β-estradiol: Sigma Chemical Co, St. Louis MO, dissolved in absolute ethanol at a concentration of 0.01 M and diluted in saline, 10^{-8} M) was administered to mice intramuscularly for 7 consecutive days. At day 7 mice were infected intra-vaginally with 10^7 IFU of MoPn per mouse. Control mice received saline only. Two and five weeks post-infection, groups of animals were mated with male counterparts by placing 3 females to 1 male, and subsequently observed and weighed daily for 19 days to determine pregnancy, as previously described (Barr *et al.* 2005). The numbers of pregnant mice in the different groups were enumerated after 19 days in each case.

Assessment of Chemokine Receptor-5 deficiency on the Infection and Immune Response during Chlamydia Infection.

In Vitro Infectivity Assay to Quantitate Chlamydial Shedding in Vaginal Washes in CCR5 deficient mice. Chemokine receptor-5 deficient mice were treated with depo-provera (25 mg/ml) seven days prior to infection to stabilize the estrus cycle. Following depo-provera treatment, mice were infected intra-vaginally with MoPn (10^7 IFU/mouse). Control mice received saline only. The course of infection was followed up to five weeks. *Chlamydiae* were isolated from the swabs in tissue culture and enumerated by

immunofluorescence according to methods described earlier. The results were repeated to include 10-12 animals per group.

Chlamydia-induced cell activation, cytokine and chemokine secretion by leukocytes, The profile of cytokines and chemokines secreted by leukocytes from chlamydial-infected CCR5KO and W/T mice was compared by measuring the levels of specific cytokines and chemokines released following *in vitro* re-stimulation of the total splenic cells with UV-inactivated chlamydiae. CCR5KO and control mice were intravaginally infected with MoPn as described above and at various times post-infection (7, 14, 21, and 28 days) splenic cells were isolated from infected mice and 2 x 10^5 cells were stimulated with chlamydial antigen (10 μg/ml) and incubated at 37 °C in 5% CO_2 for 120 h. At the end of the incubation period, the supernatants were collected and assayed for cytokines, IFN-γ, IL-4, IL-10, TNF-α, IL-1β, IL-6 and IL-8) and Th1 chemokines (RANTES, MIP-1α, MIP-1β, IP-10) using a quantitative ELISA. The concentration of the cytokine and chemokines in each was obtained by extrapolation from a standard calibration curve generated simultaneously. Data were calculated as the mean values (± standard deviation) of triplicate cultures for each experiment. The results were derived from at least 3 independent experiments.

Evaluation of CCR5 deficiency on fertility during *chlamydia* infection. Chemokine receptor-5 deficient female mice and wild type mice were infected with 10^6 IFU/mouse with MoPn. Two and five weeks post-infection, groups of animals were mated with male counterparts by placing 3 females to 1 male, and subsequently observed and weighed daily for 19 days to determine pregnancy, as previously described (Barr *et*

al. 2005). The numbers of pregnant mice in the different groups were enumerated after 19 days in each case.

Data Analysis. Quantitative data (IFUs/ml), ELISA cytokine levels, optical densities, etc.) and non-quantitative (positive and negatives) were processed and statistically analyzed where applicable. Statistical tests peformed include Fisher's exact test and student's *t* test (one and two-tailed). The relationship between different experimental groupings was assessed by analysis of variance (ANOVA). These were all available through SigmaStat statistical software (SigmaStat, San Jose, CA). Minimal statistical significance was judged at $p < .05$.

CHAPTER 4

RESULTS

The challenge to develop an efficacious vaccine for controlling genital chlamydia infections requires significant advances in several key areas of immunobiology during chlamydial infection. For instance, the understanding of immune effectors that assist in pathogen clearance and identification of factors capable of optimizing genital mucosa immune responses against an infection are needed (Igietseme *et al.* 2002). A robust and sustained T helper type I (Th1) response is considered necessary for the clearance and long term protection against genital chlamydia infection. Therefore, it is imperative to understand those factors that may act as immunosuppressors, thereby affecting the Th1 response during infection. Estrogen and chemokine receptors may play a vital role in the activation and migration of T cells to the site of infection. However, the cellular and molecular basis of these regulatory functions remains questionable. The goal of these studies was to evaluate the role of estrogen (17β-estradiol) and chemokine receptor 5 (CCR5) on the induction of protective immunity during Chlamydial infection. Using a reliable *in vitro* and murine model of chlamydial genital infection, genetically engineered specific gene knockout mice, and molecular, immunological, biochemical techniques the following study objectives included: **1)** Assessing the effect of estrogen on infectivity and chemokine production in an *in-vitro* system; **2)** Evaluating estrogen's effect on the course of infection and the induction of the protective immune response in a murine model; **3)**

Investigating the importance of CCR5 in protective immunity during Chlamydia infection; and; **4)** Assessing the role of CCR5 in pathogenesis during chlamydia infection as it relates to fertility. The results obtained are exciting and revealing, with crucial findings and paradigm shifts that are likely to enhance vaccine design against *C. trachomatis* and as well as other intracellular pathogens.

Evidence that Estrogen influences Chlamydial Infection and Immunomodulatory Molecules in a Murine *In Vitro* System

Estrogen increases chlamydial infectivity in murine epithelial cells. Studies have shown that estrogen may increase host susceptibility to chlamydia infection. It was hypothesized that estrogen pre-treatment would enhance chlamydia infectivity in epithelial cells. McCoy cells were pretreated with 17 β-estradiol (10^{-8}M) 24 hours prior to MoPn infection (10^{7} IFU/ml). Results in Figure 3 show that there was a significantly higher number of fluorescent inclusion forming units (IFU/ml) observed in the estrogen treated cells compared to the untreated ($\log_{10}3$ versus $\log_{10}1$; $p< 0.01$) cells.

Figure 4 is a visual representation of the inclusion body containing cells. Panel A represents cells without estrogen or Chlamydia; Panel B represents cells that were not treated with estrogen prior to infection. Panel C represents cells that were pretreated with estrogen and infected with MoPn 24 hours following estrogen treatment. There was a higher number of inclusion bodies observed in the estrogen treated/chlamydia infected cells (Panel C) compared to the untreated (Panel B).

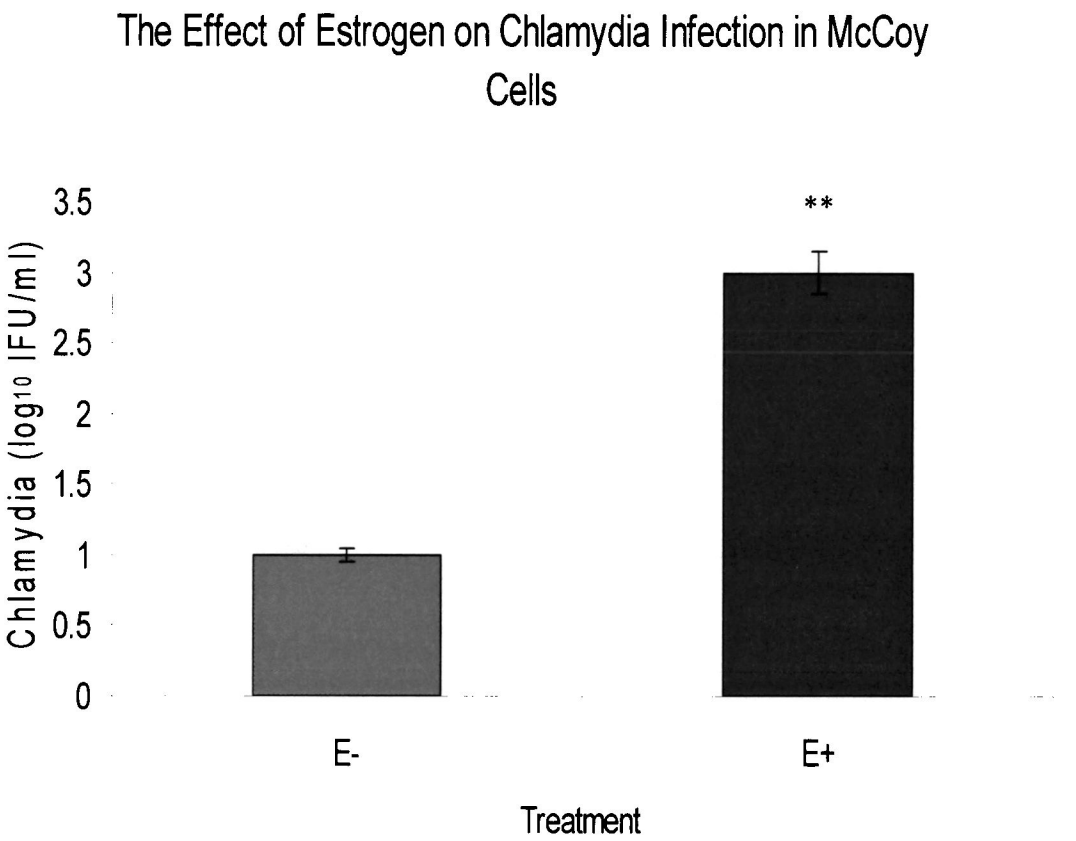
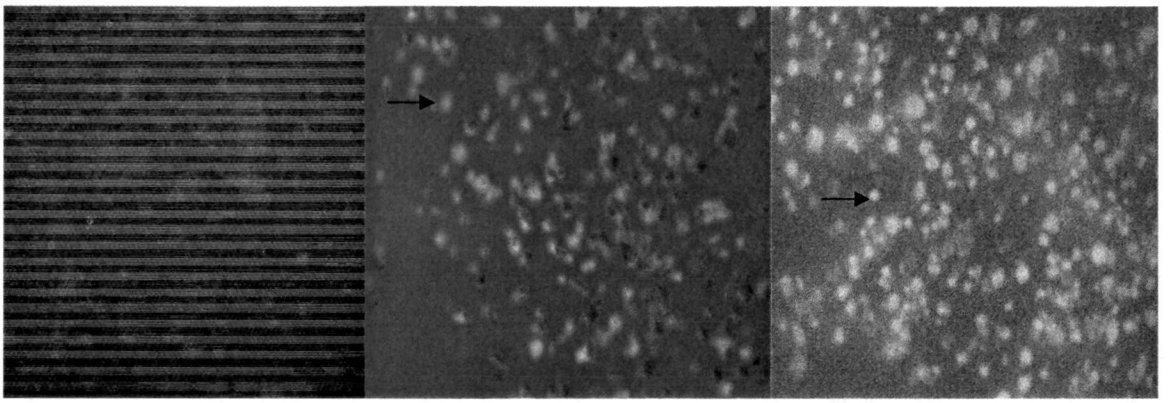


Figure 3. Quantitative analysis of estrogen's effect on chlamydia infectivity. McCoy cell monolayers were treated with 17 β-estradiol (10^{-8}M) 24 hours prior to chlamydia infection (10^7 IFU/ml) (*E+ blue*). Estrogen caused an increase in chlamydia infectivity compared to the untreated infected cells (*E-, red*) ($p < 0.01$).



A. Control **B. Chlamydia only** **C. Chlamydia and Estrogen**

Figure 4. Analysis of estrogen effects on chlamydia infectivity in McCoy cells. Immunoflorescence studies show a increase number of inclusion bodies (yellow fluorescence, arrow) were observed in the infected cells pretreated with estrogen (Panel C), compared to the untreated cells (Panel B). Panel A represents cells that were non-infected and without estrogen treatment (40 x magnification).

Estrogen causes modulation of chemokine mRNA expression in Chlamydia infected epithelial cells. Chemokines are small molecular weight proteins that attract leukocytes to the site of infection. This usually occurs during the innate immune response against chlamydia infection. To determine the effects of estrogen on chemokine mRNA expression of infected cells, quantitative and semi-quantitative PCR was performed. Quantitative PCR results established the upregulation of chemokines MIP-1α, MIP-1β and IP-10 in chlamydia infected McCoy cells. Figure 5 represents an agarose gel of the PCR amplified product. Lanes 1-3 represent the expression of MIP-1α (Lane 1), MIP-1β (Lane 2) and IP-10 (Lane 3) in the uninfected cells. Lanes 4-6 represent the expression of MIP-1α (lane 4), MIP-1 β (lane 5), and IP-10 (lane 6). There was a fold-enhancement in the mRNA expression of MIP-1α (3.86) and IP-10 (5.12) (Figs. 6-7).

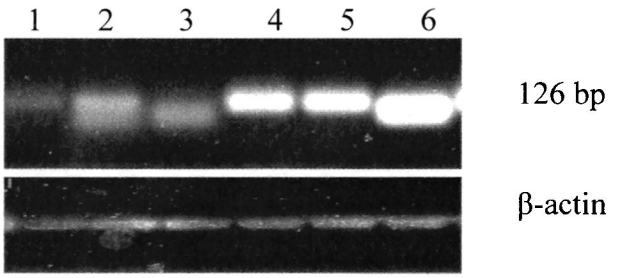


Figure 5. PCR optimization of MoPn infected McCoy cells. There was an induction of expression of MIP-1α in MoPn treated cells compared to the uninfected. (Lanes 1, MoPn infected; Lane 4 uninfected), also caused a upregulation of MIP-1β (Lane 2) compared to the uninfected cells (Lane 5). IP-10 was also upregulated in the MoPn infected (Lane 6) cells compared to the uninfected (Lane 3).

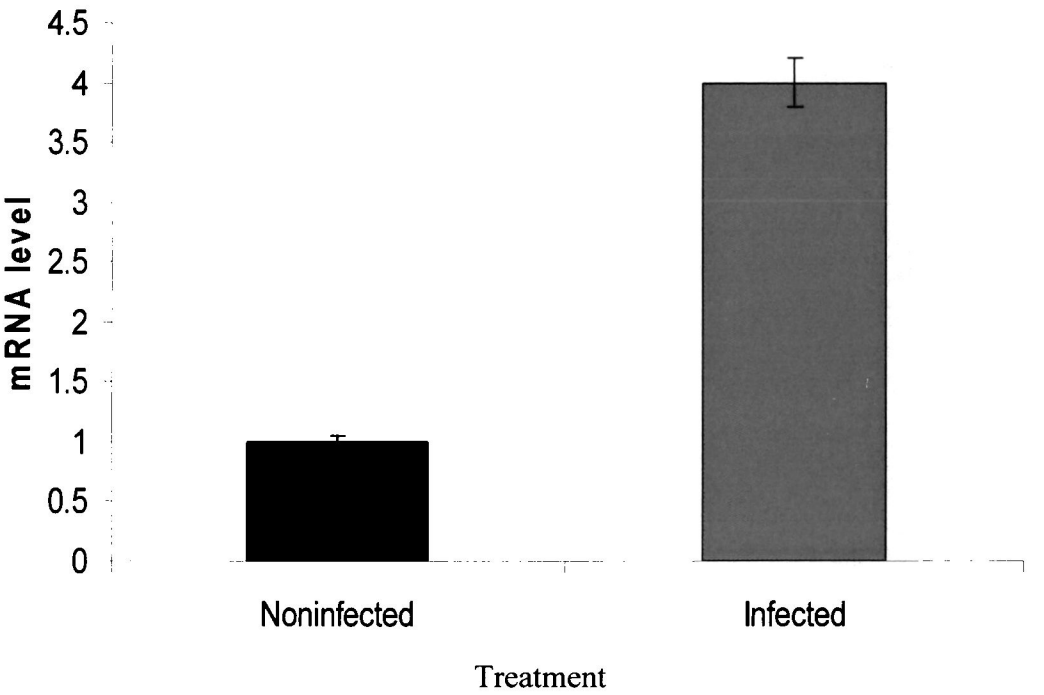


Figure 6. The effect of chlamydia infection on MIP-1α expression. McCoy cells were treated with estrogen (10^{-8} M) prior to chlamydia infection (10^{7} IFU/ml). Total RNA isolated from cells 32 hours post infection were analyzed by quantitative RT-PCR to assess the expression of level of MIP-1 α. PCR results indicated that there was a 3.86 fold increase in the infected cells *(red)* compared to the non-infected cells *(blue)*.

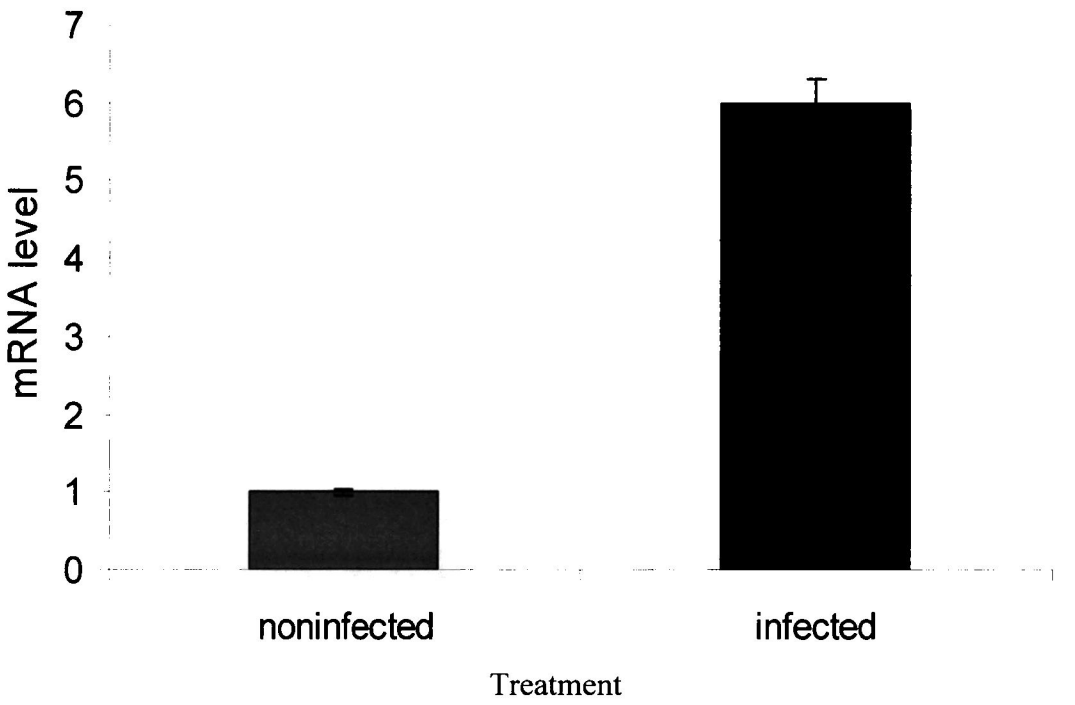


Figure 7. The effect of chlamydia infection on IP-10 expression. McCoy cells were treated with estrogen (10^{-8} M) prior to chlamydia infection (10^{7} IFU/ml). Total RNAs isolated from cells 32 hours post infection were analyzed by quantitative RT-PCR to assess the expression of level of IP-10. PCR results indicated that there was a 5.12 fold increase in the infected cells *(red)* compared to the non-infected cells *(blue)*.

Semi-quantitative results indicated that treatment of McCoy cells with estrogen followed by chlamydia infection caused a decrease in the expression of chemokines. MIP-1α expression was down-regulated in the estrogen treated, infected cells (lane 1) compared to the non-treated cells (lane 3) (Fig. 8A). The plots of the densitometric scans of the data shown in Fig. 8B, was normalized to β-actin expression and analyzed simultaneously at each point. Interestingly, estrogen pretreatment did not cause a significant change ($p > 0.02$) in the expression of MIP-1β in McCoy infected cells treated with estrogen (Fig. 9A; Lane 1) compared to the non-treated cells (Fig. 9A; Lane 3). Results in Fig. 10 shows RANTES expression in the chlamydia non-treated McCoy cells (Lane 2). There was a down-regulation of mRNA expression as shown in Lane 3. Overall, estrogen caused a decrease in mRNA expression of RANTES, MIP-1α and IP-10; these chemokines play significant roles in the induction of Th 1 cell recruitment and activation.

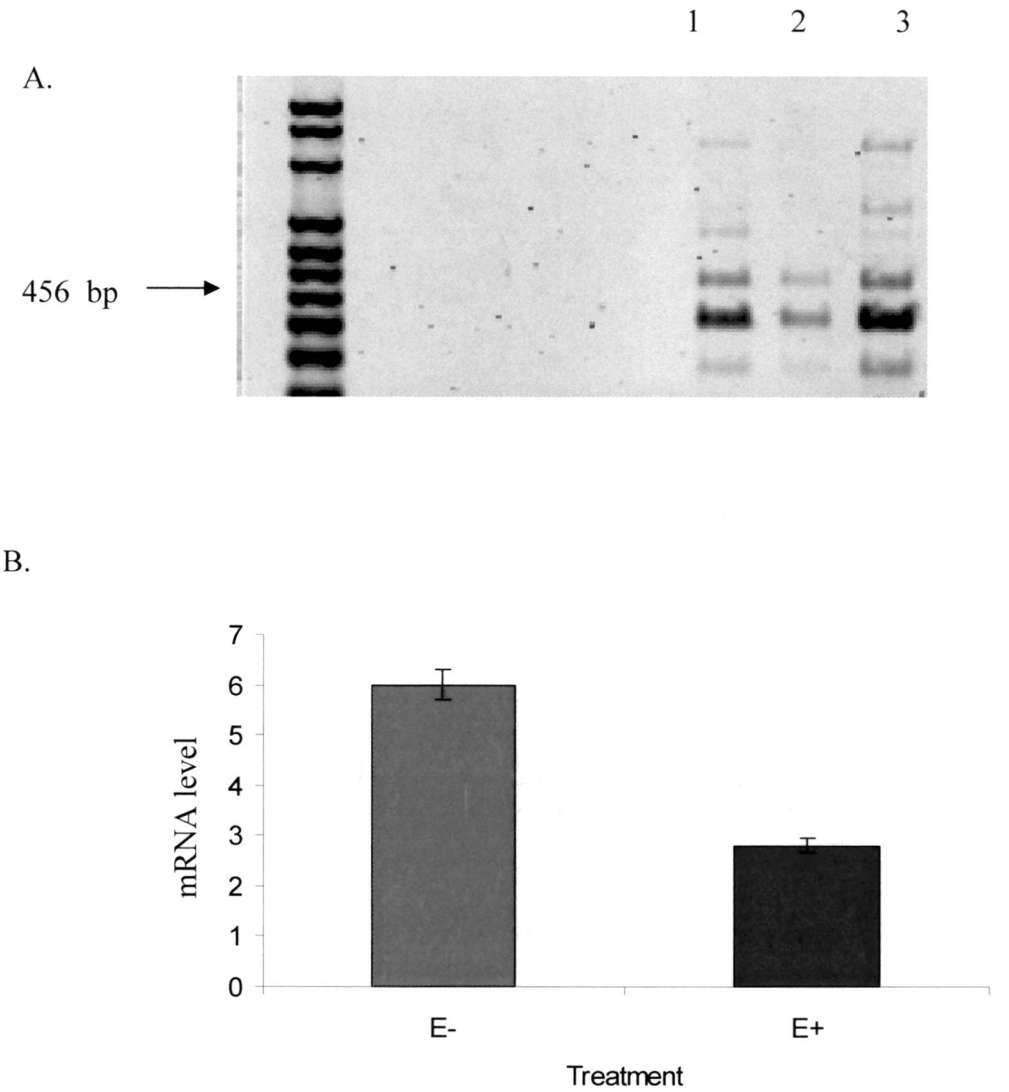
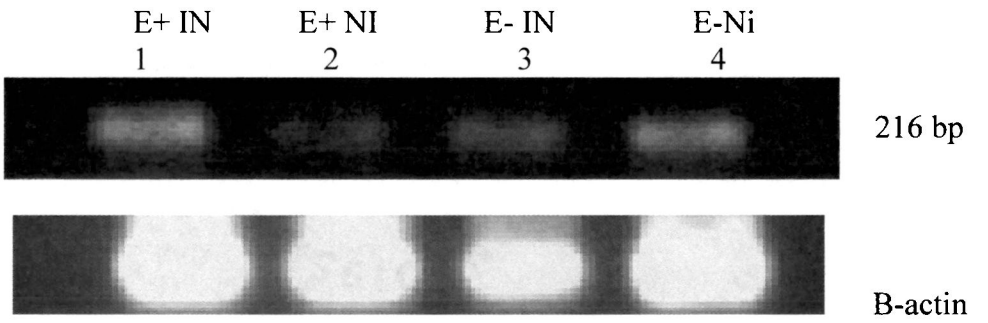


Figure 8. The effect of estrogen on mRNA expression of MIP-1α in McCoy cells. McCoy cells were pretreated with 17-β estradiol (10^{-8} M) 24 hours prior to MoPn (10^{7} IFU/ml). Total RNAs isolated from cells 32 hours post infection were analyzed by semi-quantitative RT-PCR to assess the level of expression of MIP-1 α. Estrogen caused a decrease in MIP-1α expression (Lane 1) compared to the non-treated infected cells (Lane 3). Lane 2 represents cells that were treated with estrogen only, there was a detectable expression. Panel B represents the plot of the densitometric scans of the data shown in panel A normalized to β-actin. E-, non-estrogen treated, chlamydia infected (blue); E+ estrogen treated, chlamydia infected (red).

A.



E+=Estrogen; In=Infected; E-=no Estrogen; Ni=noninfected

B.

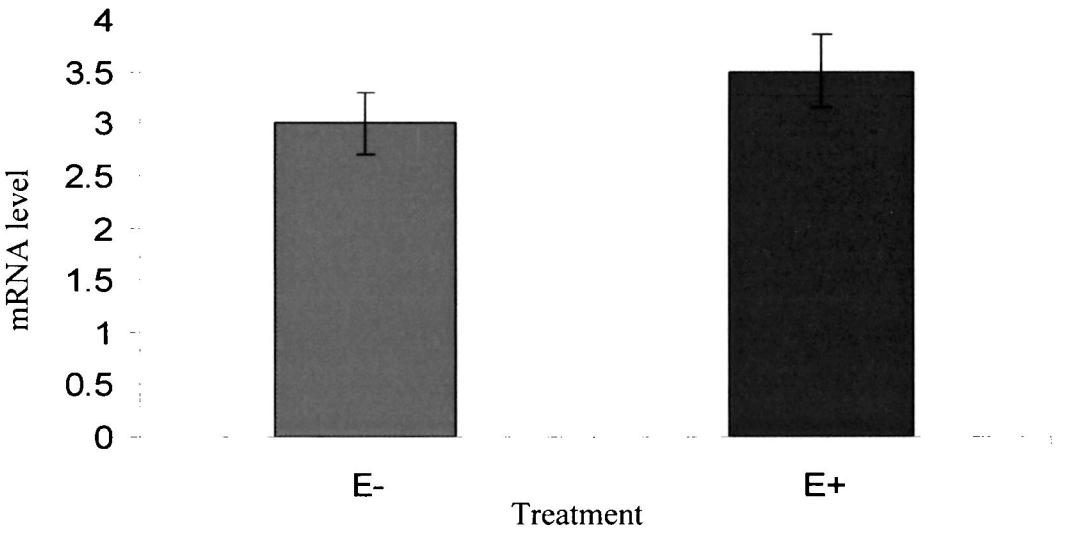
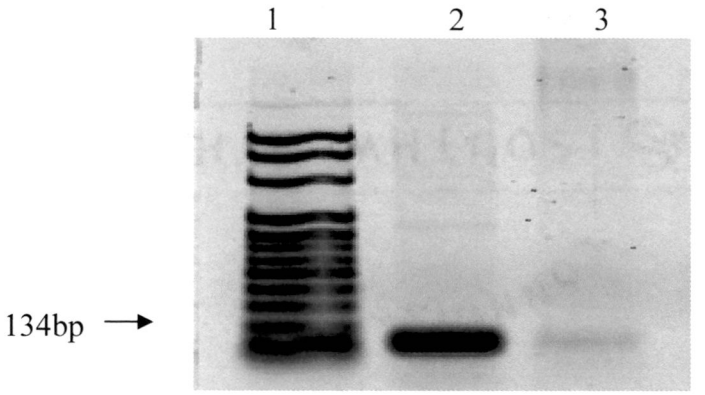


Figure 9. The effect estrogen on mRNA expression of MIP-1β in McCoy cells. McCoy cells were pretreated with 17-β estradiol (10^{-8} M) 24 hours prior to MoPn (10^{7} IFU/ml). Total RNAs isolated from cells 32 hours post infection were analyzed by semi-quantitative RT-PCR to assess the expression of level of MIP-1β. Estrogen did not cause a change in MIP-1β expression (*Lane 1*) compared to the non-treated infected cells (*Lane 3*). Panel B represents the plot of the densitometric scans of the data shown in panel A normalized to β-actin. *E-, non-estrogen treated, chlamydia infected (blue*); *E+ estrogen treated, chlamydia infected (red).*

A.



B.

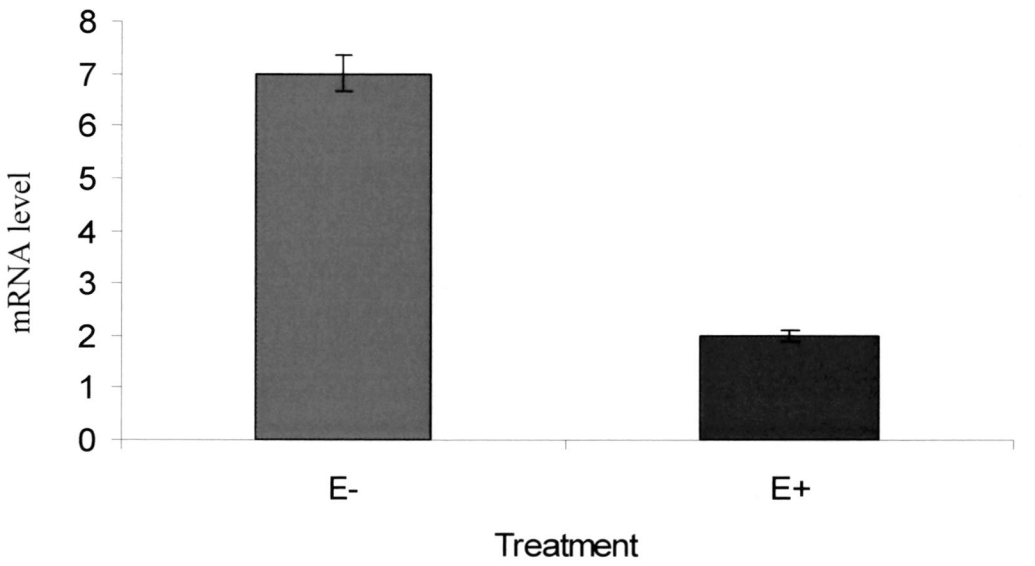


Figure 10. The effect of estrogen on RANTES mRNA expression in MoPn infected McCoy cells. McCoy cells were pretreated with 17-β estradiol (10^{-8} M) 24 hours prior to MoPn (10^{7} IFU/ml). Total RNAs isolated from cells 32 hours post infection were analyzed by semi-quantitative RT-PCR to assess the expression of level of MIP-1 α. Estrogen caused a decrease in RANTES expression (Lane 2) compared to the non-treated infected cells (Lane 3). Panel B represents the plot of the densitometric scans of the data shown in panel A normalized to β-actin. E-, non-estrogen treated, chlamydia infected *(blue);* E+ estrogen treated, chlamydia infected *(red).*

Estrogen causes modulation of chemokine production in Chlamydia infected epithelial cells. *In vitro* studies indicated that estrogen increased chlamydia infectivity in epithelial cells and caused a decrease in chemokines MIP-1α, RANTES and IP-10 mRNA expression. The purpose of these studies was to use ELISA to determine the effects of estrogen on chemokine secretion in chlamydia infected epithelial cells. Cells were treated with estrogen (10^{-8} M) for 24 hrs prior to chlamydia infection (10^{7} IFU/ml). Supernatant was collected 32 hours post infection and used for ELISA. Estrogen caused a significant ($p < 0.01$) decrease in the production of MIP-1α and a similar trend was observed in RANTES and MCP-1 production (Figs. 11-13). Estrogen did not cause a change in IP-10 production (Fig. 14). Overall, the greatest fold decrease was observed in MIP-1α, RANTES and IP-10 (Fig. 15).

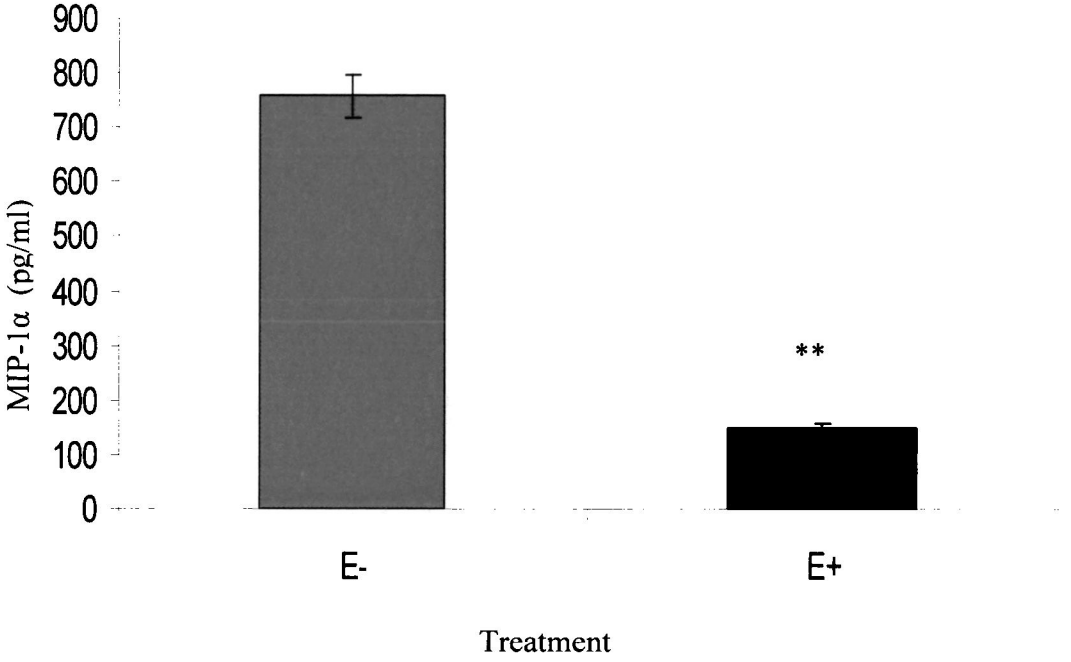


Figure 11. The effect of estrogen on MIP-1α production during Chlamydia infection. Cells pretreated with estrogen (10^{-8} M) 24 hours prior to MoPn infection resulted in lower production of MIP-1α. ELISA was performed on the supernatant collected from cells 32 hours post infection. Estrogen caused a decrease in MIP-1α production compared to the non-treated. Standard deviation from 3 experiments. *E-, no estrogen treatment, chlamydia infected (Red)*; *E+, estrogen treated, chlamydia infected (blue)*. ** $p < 0.01$

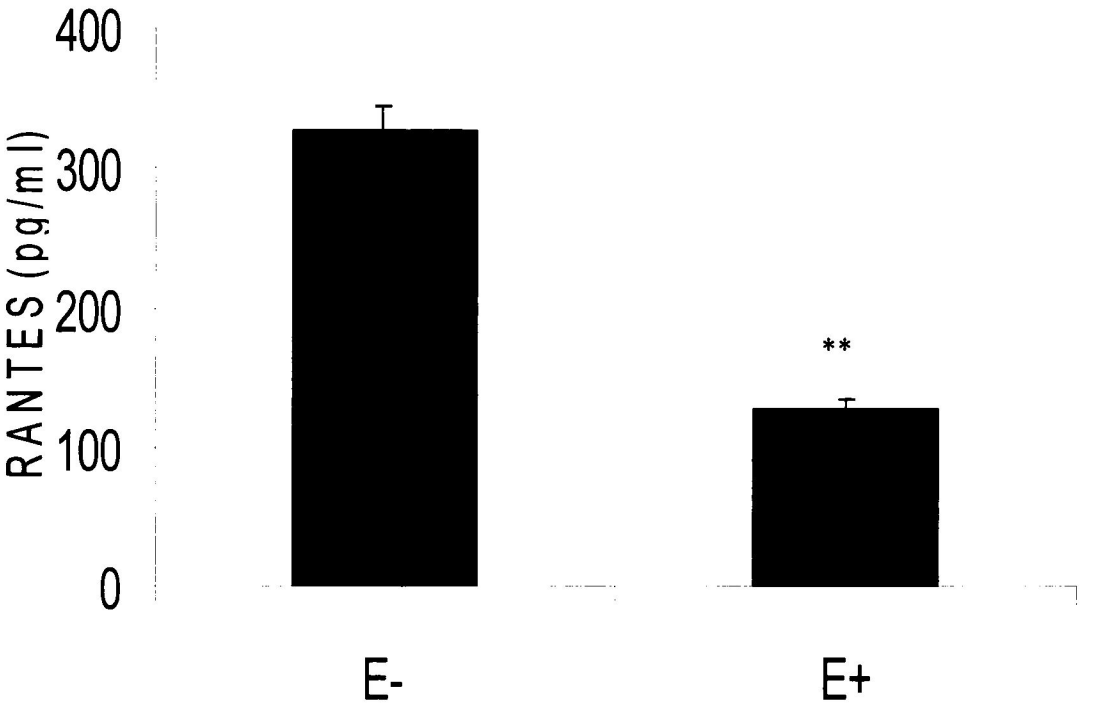


Figure 12. Effects of estrogen on RANTES production during Chlamydia infection. Cells pretreated with estrogen (10^{-8} M) 24 hours prior to MoPn infection resulted in a reduced secretion of RANTES. ELISA was performed on the supernatant collected from cells 32 hours post infection. Estrogen caused a decrease in RANTES production compared to the non-treated. Standard deviation from 3 experiments. E-, no estrogen treatment, chlamydia infected *(Red);* E+, estrogen treated, chlamydia infected *(blue).*
**$p < 0.01$

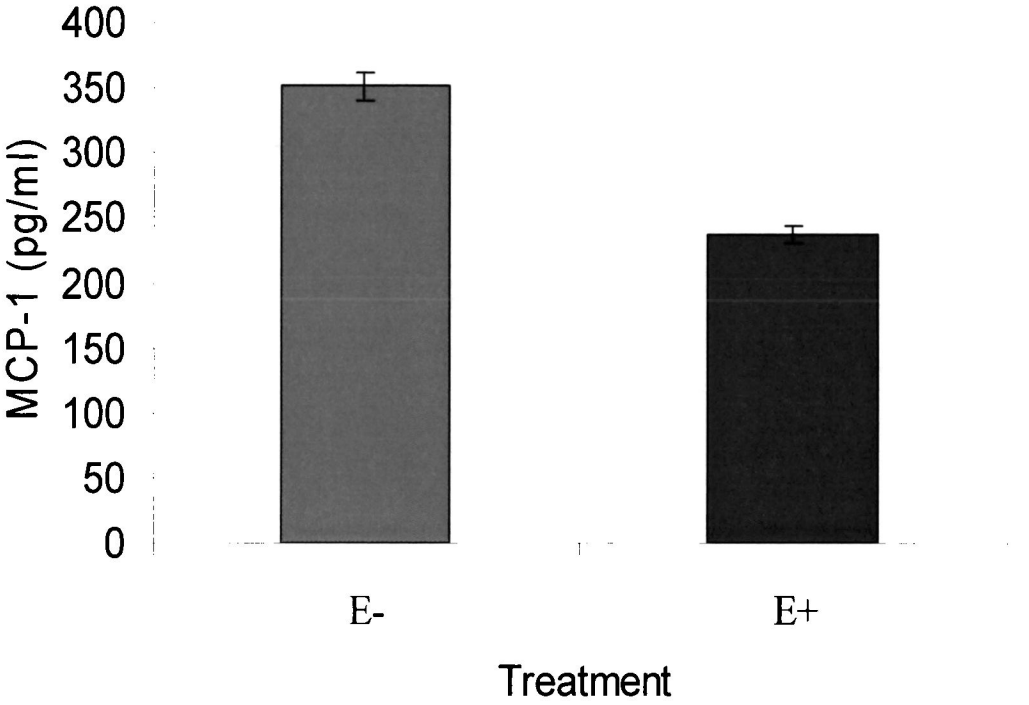


Figure 13. The effect of estrogen on MCP-1 production during Chlamydia infection. Cells pretreated with estrogen (10^{-8} M) 24 hours prior to MoPn infection resulted in secretion of MCP-1. ELISA was performed on the supernatant collected from cells 32 hours post infection. Estrogen caused a decrease in MCP-1 production compared to the non-treated. Standard deviation from 3 experiments. E-, no estrogen treatment, chlamydia infected (Red); E+, estrogen treated, chlamydia infected. *$p < 0.05$

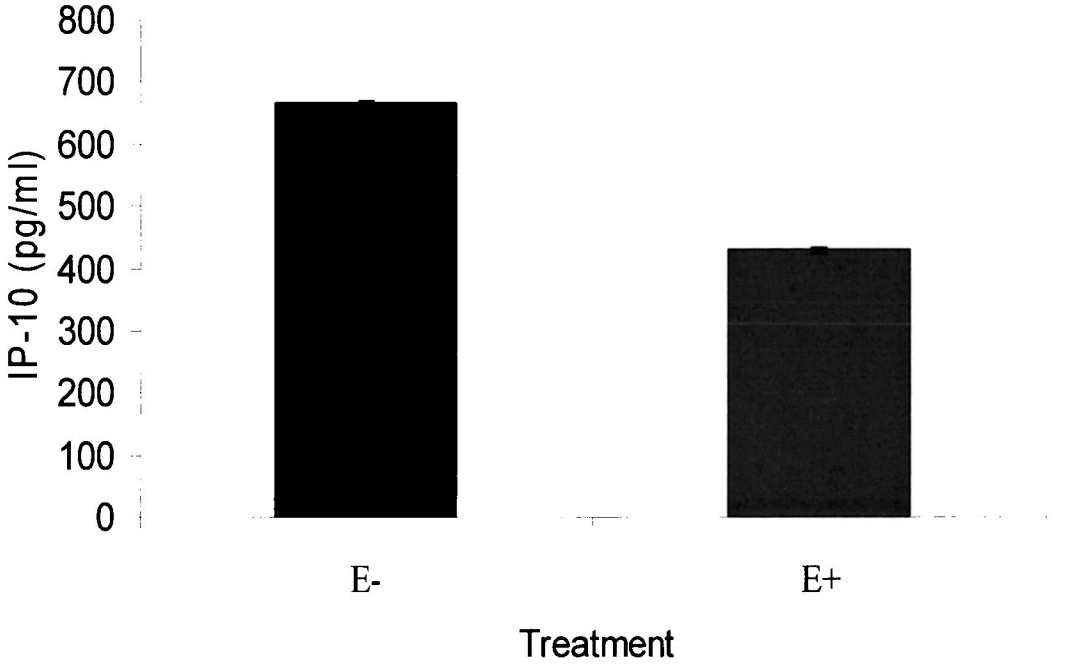


Figure 14. The effect of estrogen on IP-10 production during Chlamydia infection. Cells pretreated with estrogen (10^{-8} M) 24 hours prior to MoPn infection did not produce significant amount of IP-10. ELISA was performed on the supernatant collected from cells 32 hours post infection. Estrogen caused a decrease in IP-10 production compared to the non-treated. *E-, no estrogen treatment, chlamydia infected (Red); E+, estrogen treated, chlamydia infected (blue).* Standard deviation from 3 experiments.

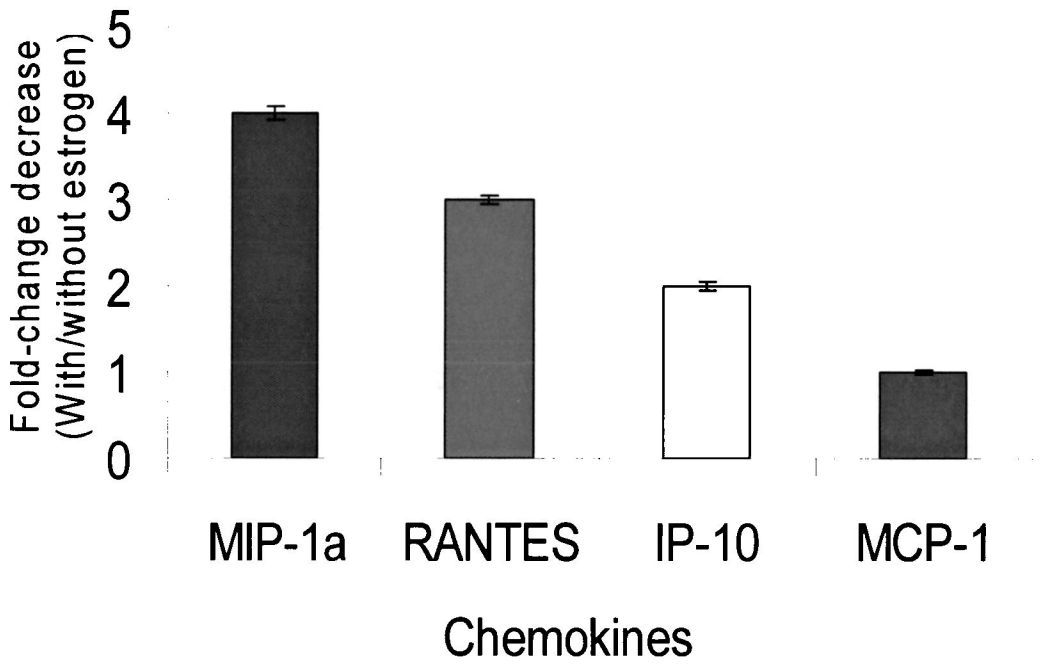


Figure 15. Effect The effect of estrogen on chemokine production during Chlamydia infection. MIP-1α and RANTES showed a greater fold decrease in the presence of estrogen.

Evaluation of the Effect of Estrogen on Course of infection and the Induction of Protective Immunity in a Murine Model

Estrogen increases cervicovaginal shedding of *C. trachomatis* infected mice. Estrogen is a sex hormone that has an effect on various immune effectors during chlamydia infection. The effect of estrogen on the ability of mice to control and clear genital chlamydial infection was investigated. Mice were treated with estrogen for 7 days prior to MoPn infection. To follow the course of infection, mice were vaginally swabbed every three days up to 30 days post infection. Chlamydia was isolated from the swabs and infectious inclusion bodies were observed and enumerated using flourescence microscopy. Results indicated that by day 12, there was a higher level of infection (log_{10} 2.5 IFU/ml versus

$\log_{10}$1.5 IFU/ml) in the estrogen treated animals compared to the non-treated animals (Fig. 16). A similar trend of infection was observed at 15, 21 and 30 days post infection. At 30 days, untreated animals resolved their infection; however there was sustained infection in the estrogen treated mice (0.0 versus $\log_{10}$1.0 IFU/ml, respectively).

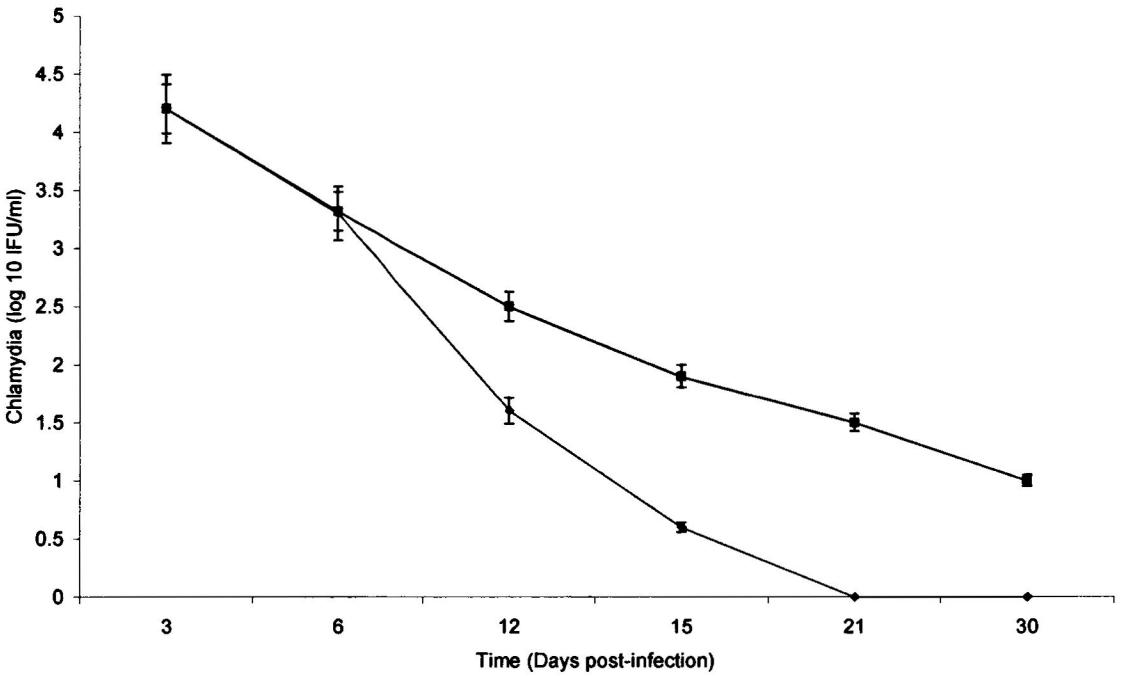


Figure 16. Effect of estrogen on Chlamydia infection in mouse model. Mice were pretreated with 17β-estradiol (10^{-8} M) prior to Chlamydia infection (10^{7} IFU) (*E-, blue*). The course of infection was followed by performing vaginal swabs. Longer duration of Chlamydia infection was observed in the estrogen treated mice compared to the untreated. (E-, non-estrogen treated, chlamydia infected *(red)*, E+, estrogen treated, chlamydia infected (*blue*). Experiments were repeated 2 times to give 10-12 mice per group.

Estrogen Causes a Decrease in Chemokine Secretion during Chlamydia Infection in a Mouse Model. The direct immunobiologic impact of estrogen may include the limitation of T cell activation and reduction or elimination of recruitment of leukocytes to the site of infection. Since these processes are mediated by chemokines and cytokines, their production in estrogen treated mice that were exposed to chlamydia was evaluated. Mice were pretreated with estrogen for 7 consecutive days prior to chlamydia infection. At various times post infection (7, 14, 21, and 38 days), the spleen was harvested. When splenic cells containing T cells and other leukocytes were exposed to chlamydia, the antigen-specific chemokine/cytokine response was decreased in the estrogen treated mice compared to the wild-type mice. MIP-1α production was significantly ($p < 0.01$) decreased after estrogen treatment at 7-38 days post infection (Fig. 16). Figure 18 indicates that estrogen caused a decrease in MIP-1β production at 7, 21 and 38 days post-infection. At 14 days, there was not a significant difference ($p > 0.02$) in MIP-1β production in the estrogen treated and non treated mice. Moreover, RANTES production was significantly lower ($p < 0.01$) in the estrogen treated mice compared to the non-treated at 7, 14 and 21 days post infection (Fig. 18). No significant difference was observed between the experimental groups at day 38. Intercellular adhesion molecule type 1 (ICAM-1) production was affected by estrogen production as showing a decrease at 7-38 days post infection (Fig. 20). Granulocyte macrophage-colony stimulating factor (GM-CSF) is responsible for the maturation of dendritic cells. Estrogen treatment caused a decrease in GM-CSF at 7, 14, 21, and 38 days post infection (Fig. 21). Collectively,

estrogen had an overall significant effect on the production of these chemokines, some of which are important in the recruitment of Th1 cells.

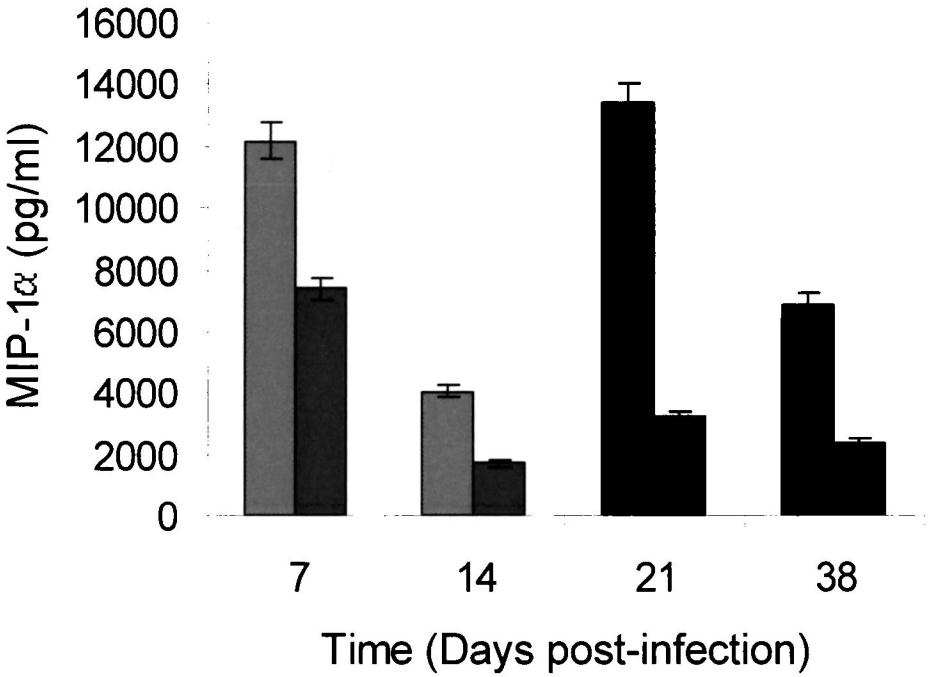


Figure 17. The effect of estrogen on MIP-1α production during genital Chlamydia infection in a mouse model. Estrogen treated(10^{-8} M; *E+, blue*) and non treated (*E-, red*) mice were infected with *Chlamydia muridarum* (the *C. trachomatis* agent of mouse pneumonitis) and at various times post-infection (7, 14, 21 and 38 days) splenic cells were isolated from infected mice and 2 x 10^5 cells were stimulated with 10 μg/ml of chlamydial antigen for 120 h. The supernatants were assayed using ELISA for MIP-1α. Estrogen caused a decrease in MIP-1α production at 7, 14, 21, and 38 days post infection. Data are mean ± standard deviation of triplicate cultures for each experiment; the results are derived from at least 3 independent experiments.

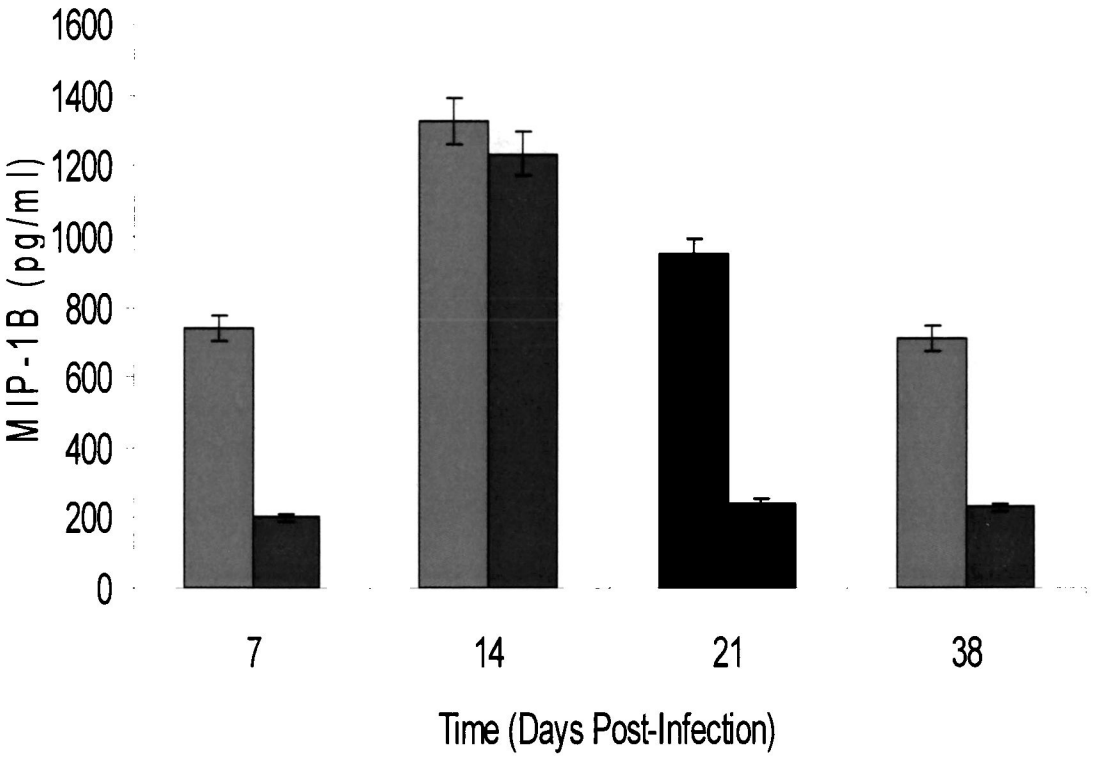


Figure 18. The effect of estrogen on MIP-1β production during genital Chlamydia infection in a mouse model. Estrogen treated (10^{-8}M; E+, blue) and non treated (E-, red) mice were infected with *Chlamydia muridarum* (the *C. trachomatis* agent of mouse pneumonitis). At various times post-infection (7, 14, 21 and 38 days) splenic cells were isolated from infected mice and 2 x 10^5 cells were stimulated with 10 μg/ml of chlamydial antigen for 120 h. The supernatants were assayed using ELISA for MIP-1β. Estrogen caused a decrease in MIP-1β production at each time point post-infection during Chlamydia infection *in-vivo*. Data are mean ± standard deviation of triplicate cultures for each experiment; the results are derived from at least 3 independent experiments.

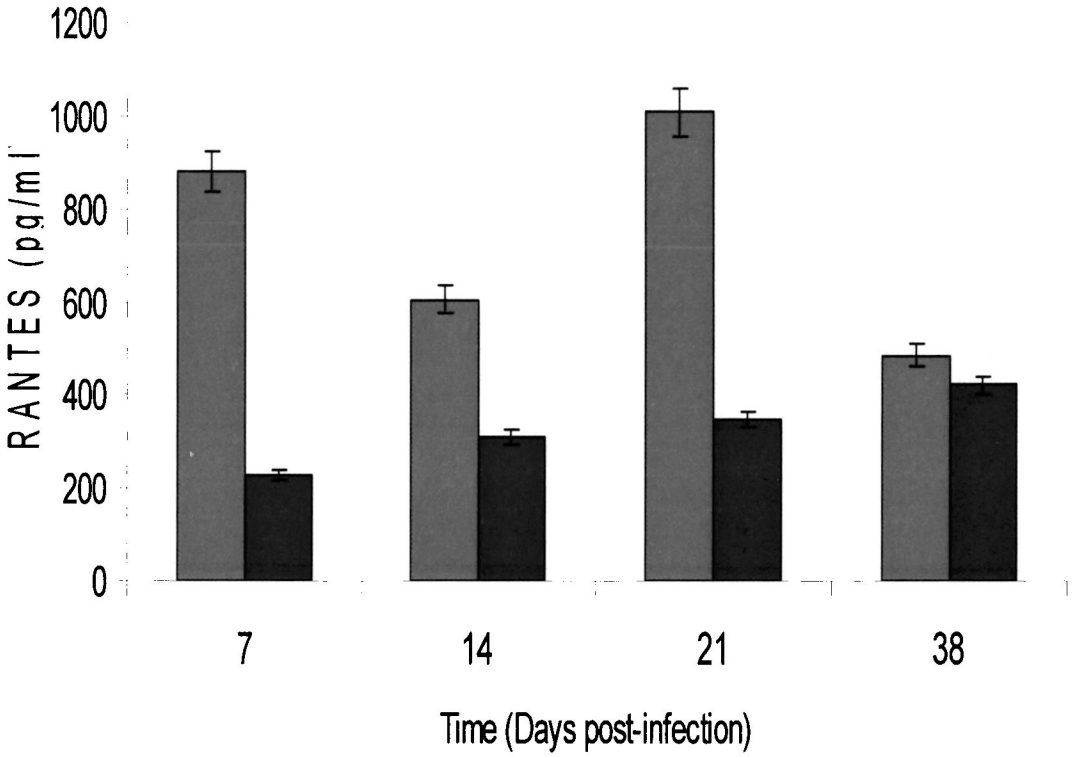


Figure 19. Effects of estrogen on RANTES production during genital Chlamydia infection. Estrogen treated (10^{-8}M; E+, blue) and non treated (E-, red) mice were infected with *Chlamydia muridarum* (the *C. trachomatis* agent of mouse pneumonitis). At various times post-infection (7, 14, 21 and 38 days) splenic cells were isolated from infected mice and 2 x 10^5 cells were stimulated with 10 μg/ml of chlamydial antigen for 120 h. The supernatants were assayed using ELISA for RANTES. Estrogen caused a decrease in RANTES production 7, 14, and 21 days post infection during Chlamydia infection *in-vivo*. Data are mean ± standard deviation of triplicate cultures for each experiment; the results are derived from at least 3 independent experiments.

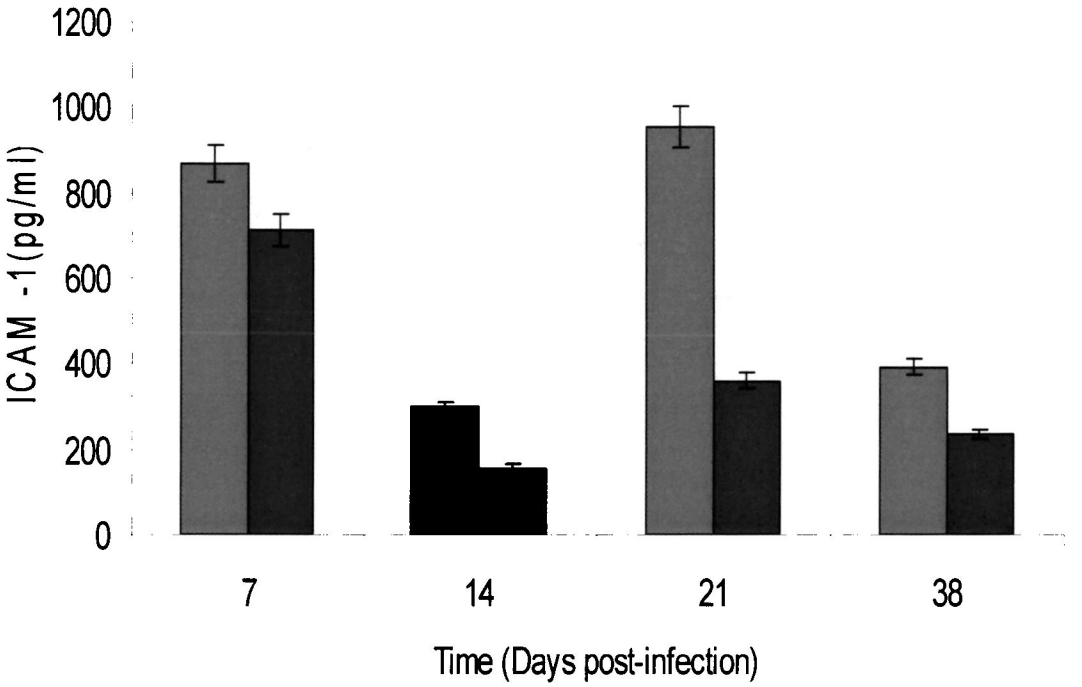


Figure 20. Effects of estrogen on ICAM-1 production during genital Chlamydia infection. Estrogen treated (10^{-8} M; E+, blue) and non treated (E-, red) mice were infected with *Chlamydia muridarum* (the *C. trachomatis* agent of mouse pneumonitis). At various times post-infection (7, 14, 21 and 38 days) splenic cells were isolated from infected mice and 2 x 10^5 cells were stimulated with 10 µg/ml of chlamydial antigen for 120 h. The supernatants were assayed using ELISA for ICAM-1. Estrogen caused a decrease in ICAM-1 production 7, 14, 21, and 38 days post infection during Chlamydia infection *in-vivo.* Data are mean ± standard deviation of triplicate cultures for each experiment; the results are derived from at least 3 independent experiments.

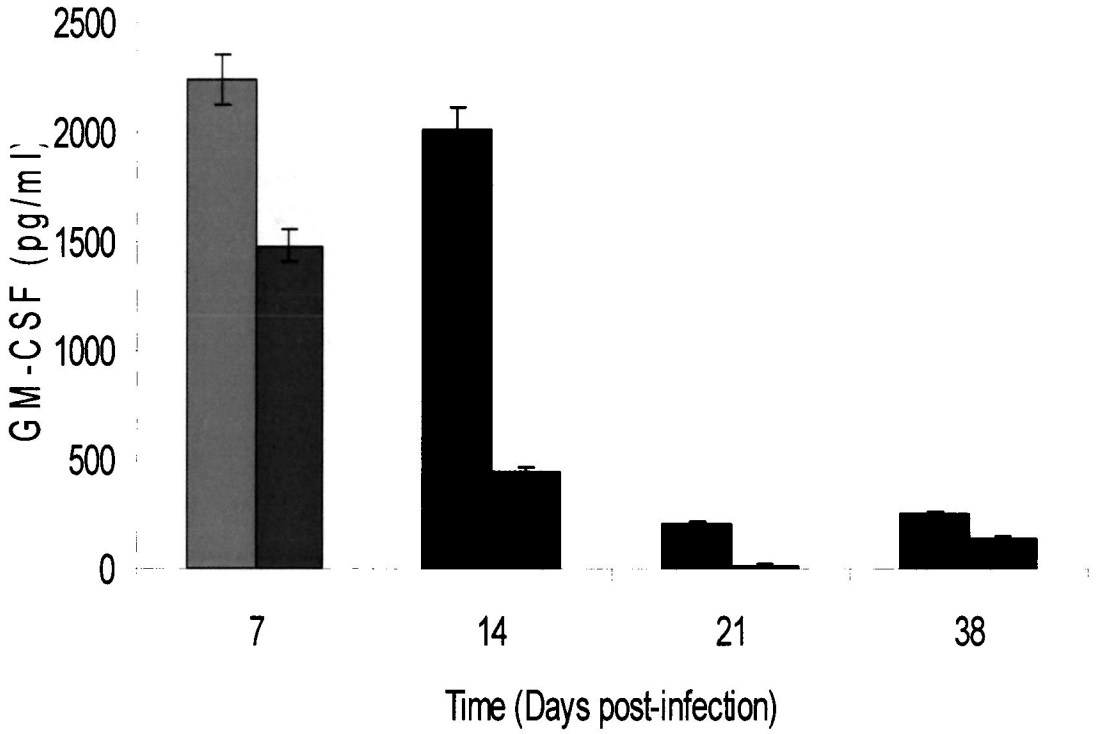


Figure 21. Effects of estrogen on GM-CSF production during Genital Chlamydia infection in a mouse model. Estrogen treated (10^{-8} M; E+, blue) and non treated (E-, red) mice were infected with *Chlamydia muridarum* (the *C. trachomatis* agent of mouse pneumonitis). At various times post-infection (7, 14, 21 and 38 days) splenic cells were isolated from infected mice and 2 x 10^5 cells were stimulated with 10 μg/ml of chlamydial antigen for 120 h. The supernatants were assayed using ELISA for GM-CSF. Estrogen caused a decrease in GM-CSF production 7,14, 21, and 38 days post-infection during Chlamydia infection *in-vivo.* Data are mean ± standard deviation of triplicate cultures for each experiment; the results are derived from at least 3 independent experiments.

Measurement of inflammatory cytokines secreted by T lymphocytes in estrogen treated mice infected with genital chlamydia. Some cytokines induce the inflammatory processes that have been associated with causing pelvic inflammatory disease, tubal scarring and infertility during chlamydia infection. When splenic cells containing T cells and other leukocytes from estrogen treated and non-treated mice were exposed to chlamydia, the antigen specific interleukin-6 (IL-6), interleukin 1α (IL-1α), tumor necrosis factor-alpha (TNF-α) and IL-12 were measured in the supernatants using quantitative ELISA. Results showed that the TNF-α level was low at 14 days post infection in the estrogen treated compared to the non treated cells (Fig. 22). IL-6 levels were progressively lower in estrogen treated animals 14, 21 and 38 days post-infection (Fig. 23). Observations indicated that IL-1α levels decreased at 7, 14, and 21 days post-infection in estrogen treated mice (Fig. 24). There was no measurable IL-1α production at 38 days post-infection. Moreover, decreased levels of IL-12 were observed in the estrogen treated animals 14 and 21 days post-infection (Fig. 25). In summation, estrogen treatment showed a trend in lowering the production of inflammatory cytokines. As mentioned earlier, the inflammatory response has been correlated to the onset of pelvic inflammatory disease and tubal factor infertility during repeated exposure to chlamydia. It was of interest to study the effects of estrogen on the pathogenesis during chlamydia infection.

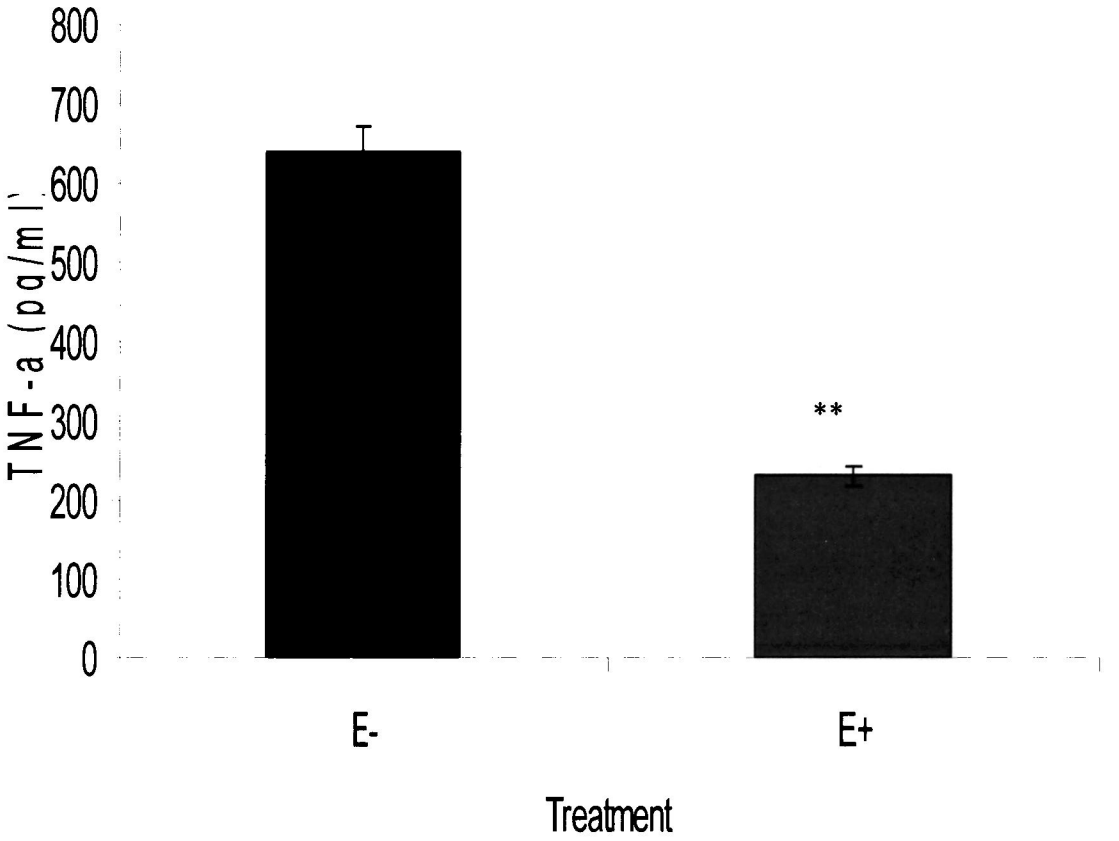


Figure 22. The effects of estrogen on TNF-α production during genital Chlamydia infection. Estrogen treated (10^{-8}M; *E+, blue)* and non treated (*E-, red*) mice were infected with *Chlamydia muridarum* (the *C. trachomatis* agent of mouse pneumonitis). At various times post-infection (7, 14, 21 and 38 days) splenic cells were isolated from infected mice and 2 x 10^5 cells were stimulated with 10 μg/ml of chlamydial antigen for 120 h. The supernatants were assayed using ELISA for TNF-α. Estrogen caused a decrease in TNF-α production 14 days post infection during Chlamydia infection *in-vivo (p 0.01).* Data are mean ± standard deviation of triplicate cultures for each experiment; the results are derived from at least 3 independent experiments.

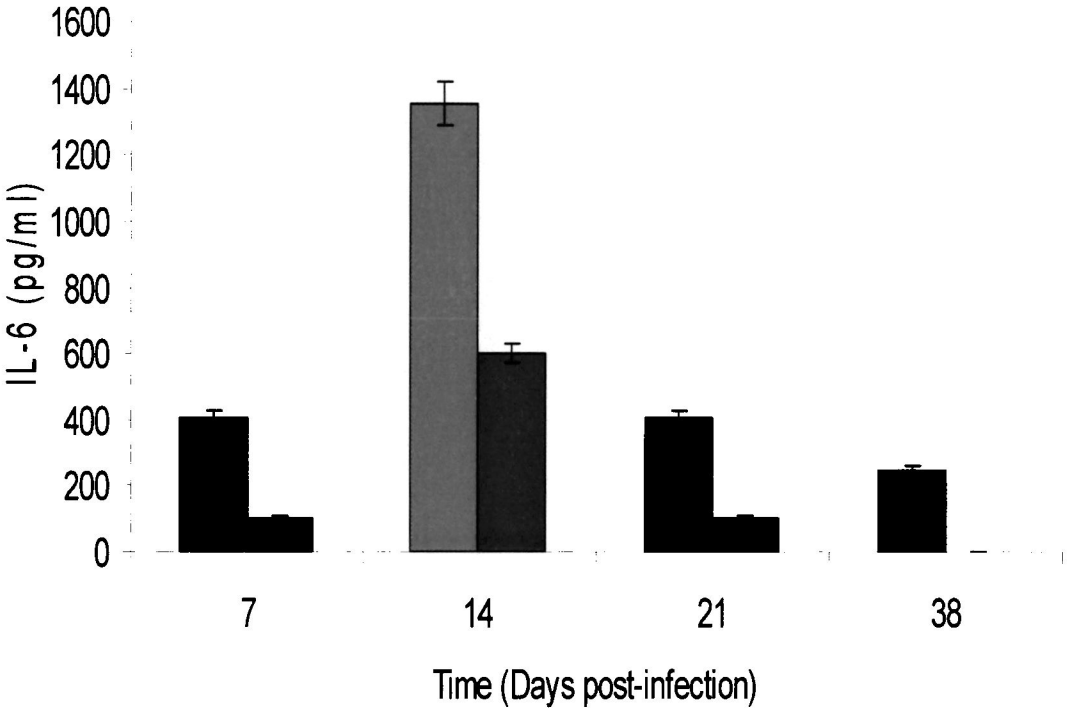


Figure 23. The effect of estrogen on IL-6 production during genital chlamydia infection. Estrogen treated (10^{-8}M; E+, blue) and non treated (E-, red) mice were infected with *Chlamydia muridarum* (the *C. trachomatis* agent of mouse pneumonitis). At various times post-infection (7, 14, 21 and 38 days) splenic cells were isolated from infected mice and 2 x 10^5 cells were stimulated with 10 μg/ml of chlamydial antigen for 120 h. The supernatants were assayed using ELISA for IL-6. Estrogen caused a decrease in IL-6 production (7, 14, 21 and 38 days postinfection) during Chlamydia infection *in-vivo.* Data are mean ± standard deviation of triplicate cultures for each experiment; the results are derived from at least 3 independent experiments.

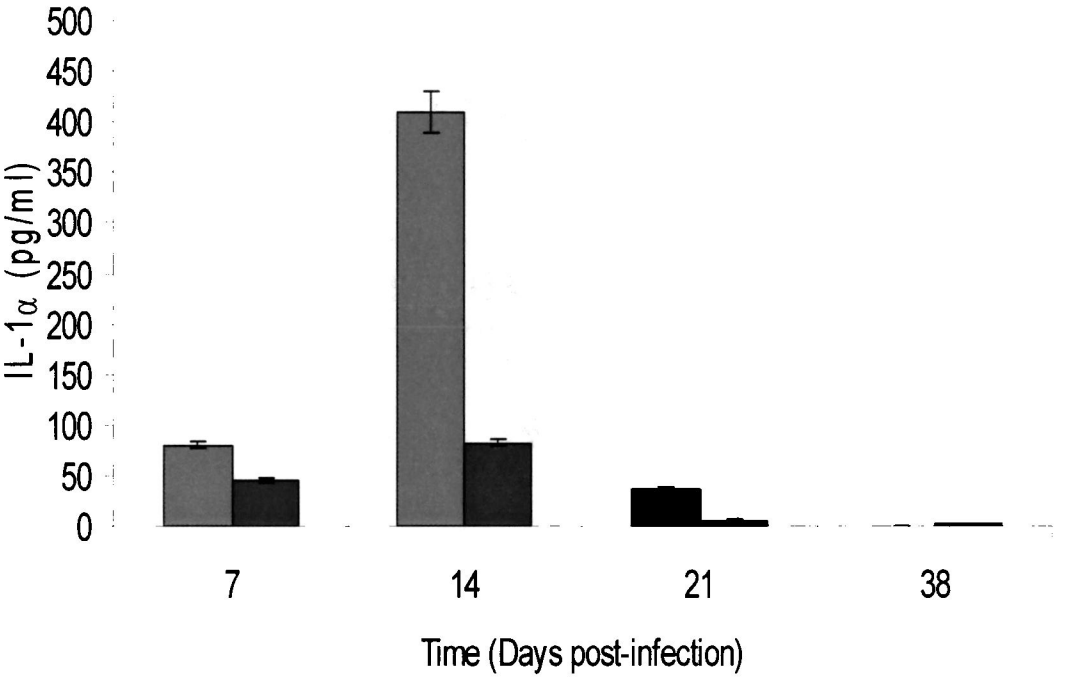


Figure 24. The effect of estrogen on IL-1α production during genital chlamydia infection. Estrogen treated (10^{-8} M; E+, blue) and non treated (E-, red) mice were infected with *Chlamydia muridarum* (the *C. trachomatis* agent of mouse pneumonitis). At various times post-infection (7, 14, 21 and 38 days) splenic cells were isolated from infected mice and 2 x 10^5 cells were stimulated with 10 μg/ml of chlamydial antigen for 120 h. The supernatants were assayed using ELISA for IL-1α. Estrogen caused a decrease in IL-1α production (7, 14 and 21 days post-infection) during Chlamydia infection *in-vivo*. Data are mean ± standard deviation of triplicate cultures for each experiment; the results are derived from at least 3 independent experiments Estrogen caused a decrease in IL-1α production (14 and 21 days pi) during Chlamydia infection *in-vivo*.

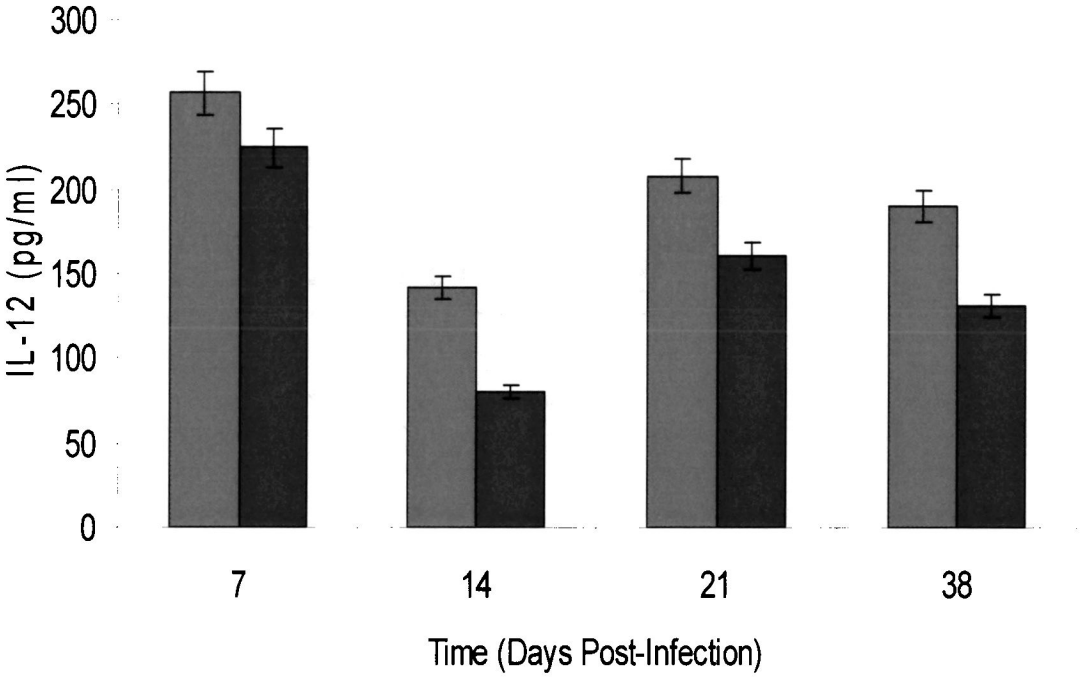
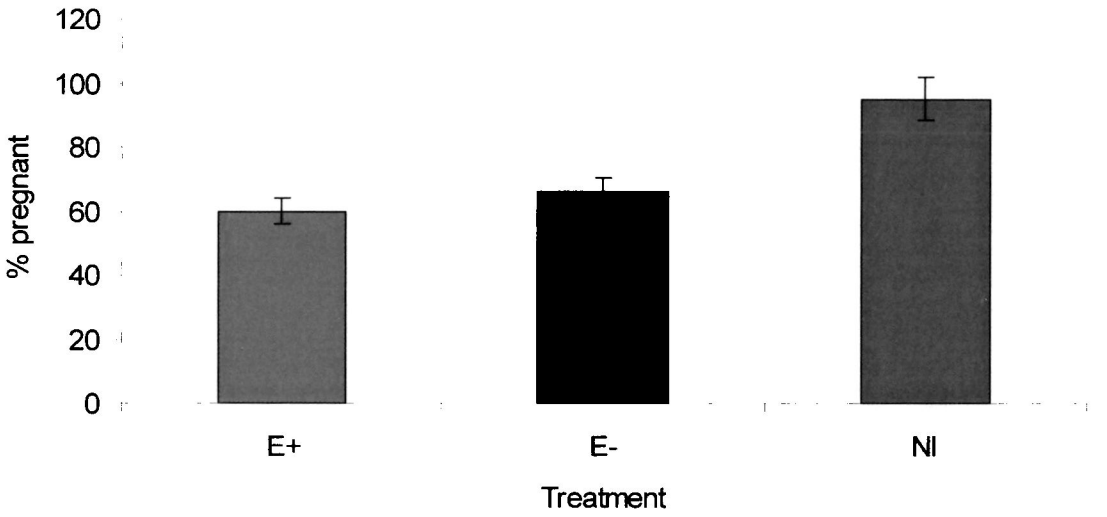


Figure 25. The effect of estrogen on IL-12 production during genital chlamydia infection. Estrogen treated (10^{-8} M; E+, blue) and non treated (E-, red) mice were infected with *Chlamydia murderer* (the *C. trachoma is* agent of mouse pneumonitis). At various times post-infection (7, 14, 21 and 38 days) splenic cells were isolated from infected mice and 2 x 10^5 cells were stimulated with 10 μg/ml of chlamydial antigen for 120 h. The supernatants were assayed using ELISA for IL-12. Estrogen caused a decrease in IL-12 production (14 and 21 days pi) during Chlamydia infection *in-vivo.* Data are mean ± standard deviation of triplicate cultures for each experiment; the results are derived from at least 3 independent experiments. Estrogen caused a decrease in IL-12 production (14 and 21 days pi) during Chlamydia infection *in-vivo.*

Measurement of fertility in Estrogen treated mice during genital Chlamydia Infection. The effect of estrogen on fertility in chlamydia infected mice was measured approximately 19 days post mating. Mice were mated early (2 weeks) and late (5 weeks) post-infection. There was no significant difference ($p > 0.02$) in the percentage of pregnancy between the estrogen treated and the non-treated (67% E- vs. 60% E+) animals

(Fig. 26A). A similar trend was observed in estrogen treated females that were mated late in infection (Fig. 26B).

A.



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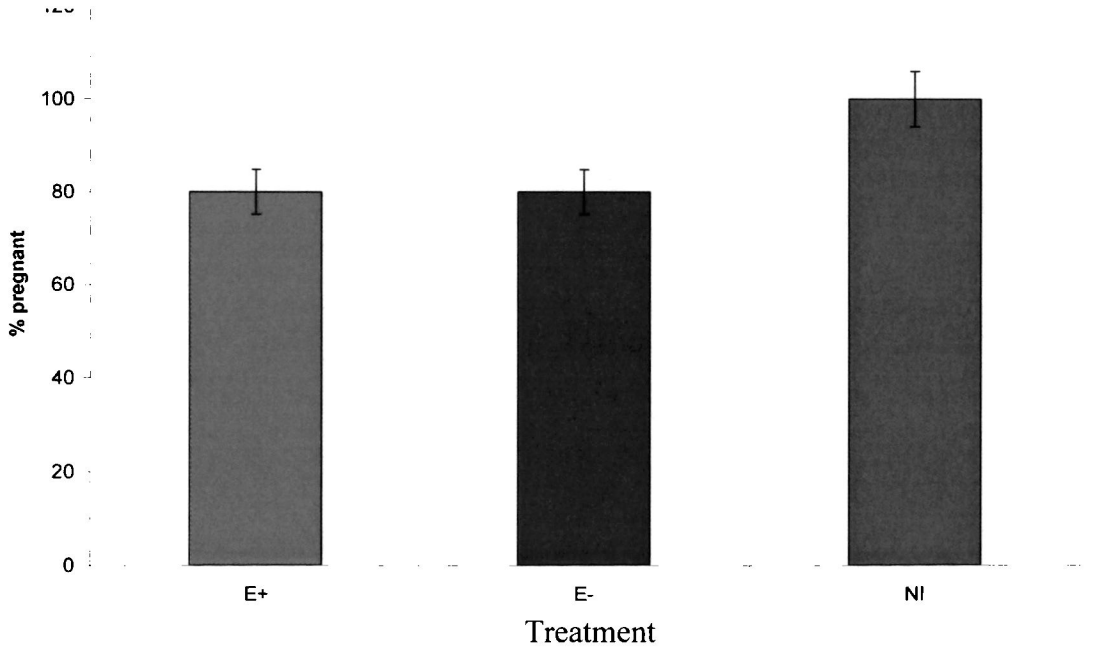


Figure 26. The effect of estrogen on fertility during genital Chlamydia infection. Female mice were treated with estrogen for 7 days prior to intravaginal infection with MoPn.

Two weeks (panel A) and five weeks (panel B) post-infection females mated with males. Nineteen days post mating females were sacrificed and evaluated for pregnancy. Estrogen did not have a significant effect on the ability of infected animals to get pregnant

Evaluation of the role of CCR5 in the immune response and pathogenesis during Chlamydia infection

High intensity of infection and delayed clearance of genital chlamydial infection in CCR5 deficient mice. CCR5 is a crucial chemokine receptor that supports the activation and induction of specific T cells during infection and non-infection inflammatory processes. We investigated the effect of CCR5 deficiency on the ability of mice to control and clear genital Chlamydial infection. Figure 27 shows results from studies that compared the course of genital chlamydial infection in the knockout ($CCR5^{-/-}$) and control (wild type, WT) mice. The data revealed that within the first week of infection, there was no difference in the ability of CCR5KO and WT mice to clear the infection. However, by the second and fourth weeks, the ability of CCR5 mice to control the infection was compromised, with a higher intensity of infection revealed by the isolation of higher chlamydiae from the mice. By the fifth week, all WT mice had cleared the infection but the CCR5KO mice remained infected (0.0 versus Log_{10} 3.0 IFU/ml, respectively). The results suggested that the deficiency of CCR5 could have adversely affected the ability of the mice to elicit the required T cell response that is known to clear chlamydiae in mice.

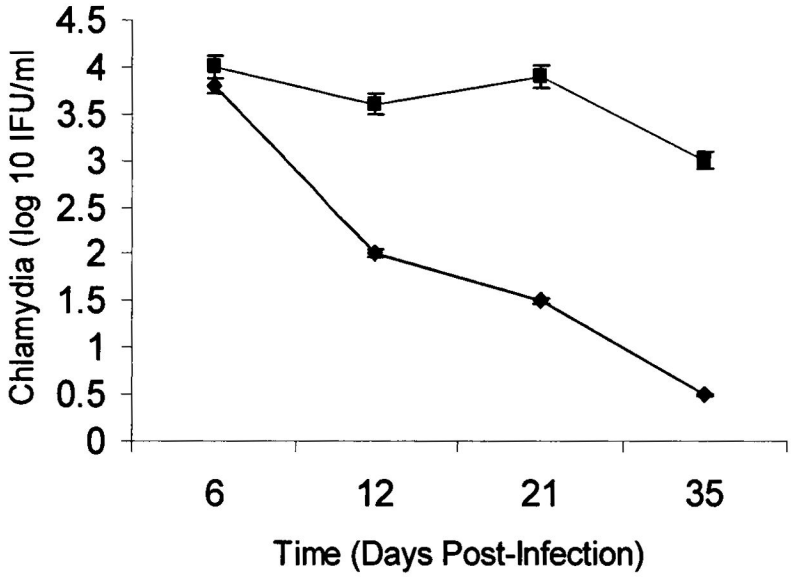


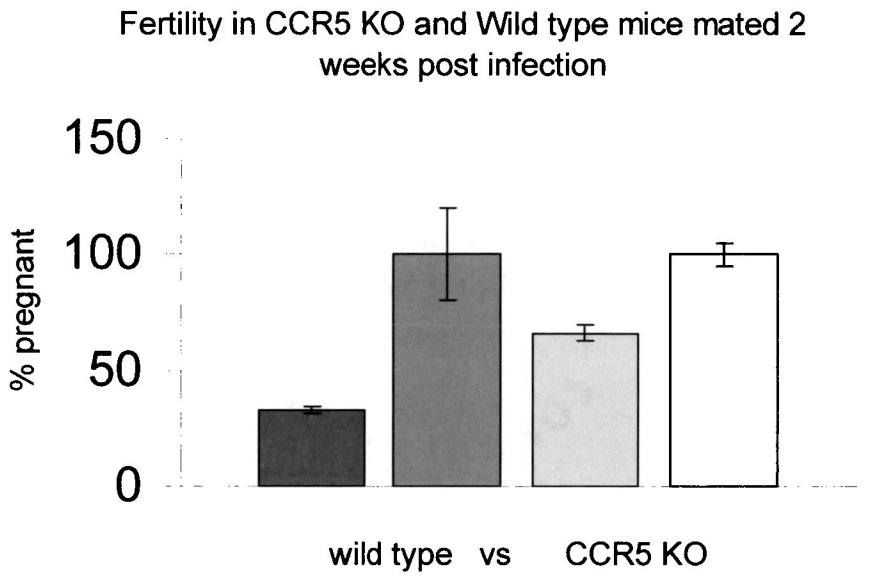
Figure 27. Isolation of Chlamydia from CCR5 KO and wild type mice. Female chemokine receptor CCR5 knockout *(red)* (CCR5KO or $CCR5^{-/-}$) and control $CCR5^{+/+}$ *(blue)* mice were intravaginally infected with 10^6 IFU of MoPn. The status of the infection was monitored by periodic cervico-vaginal swabbing of individual animals and isolation of chlamydiae in tissue culture. Experiments were repeated 2 times to give 10-12 mice per group.

Protection of CCR5 deficient mice from certain complications of chlamydial infection

Repeated exposure to *C. trachomatis* has been known to cause ectopic pregnancy and fallopian tube scarring leading to infertility in women. The absence of CCR5 will delay the Th 1 response responsible for the clearance of chlamydia which may result in ascending infection, thereby causing the previously mentioned symptoms. The effect of the diminished capacity of CCRKO mice to clear genital chlamydial infection on the infertility that is commonly associated with a genital infection was studied. Infected mice were mated at two and five weeks after the initial infection, and the fertility was assessed

by the number of pregnancies recorded. The mating at different time periods was targeted at evaluating the short- and long-term effect of the infection on fertility, since the WT mice cleared their infection at this latter time. Interesting, at 2 weeks post genital infection, WT mice exhibited a significantly lower fertility (with < 40% pregnancy rate) than CCR5KO mice ($\geq$ 70%; $p > 0.021$) (Figure 22, panel A). Furthermore, at 5 weeks post genital infection, all the CCR5KO mice exhibited 100% fertility whereas the control mice scored approximately 50% (Figure 22, panel B). In addition, CCR5KO had a higher number of pups *in utero* compared to the wild-type (Table 1). These results suggested that the immunocompetence of the host is possibly a relevant factor in the development of the long-term complication of chlamydial infection such as infertility. So we evaluated the likely immune correlates of this inverse relationship between the ability to clear chlamydial infection and the development of complications.

A.



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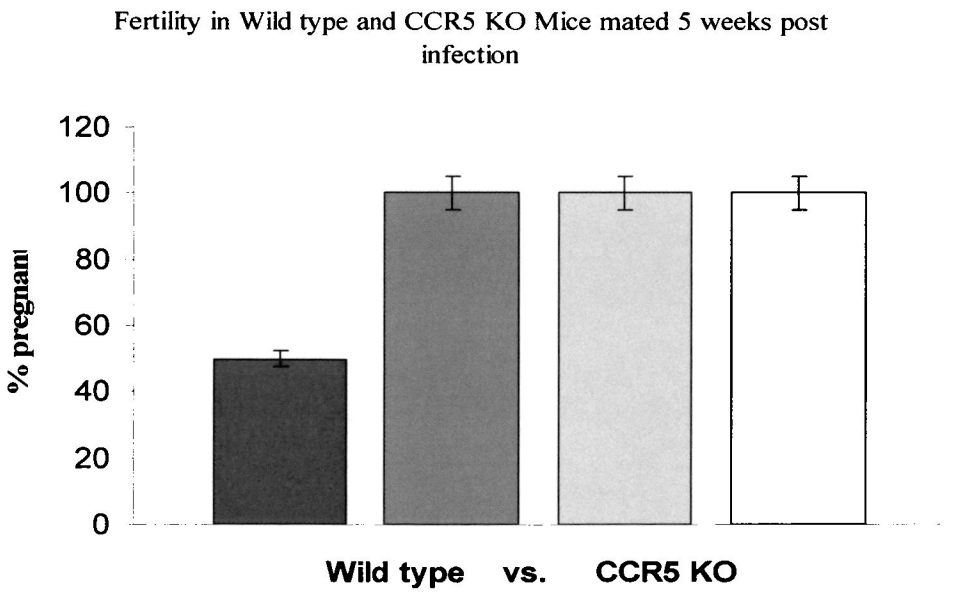

Figure 28. Fertility in CCR5 KO and wild type mice during genital Chlamydia infection. Animals were infected with 10^6 IFU of the MoPn. Two (A) and Five (B) weeks post infection, groups of animals were mated with males, and subsequently observed for 19 days to determine pregnancy. The numbers of pregnant mice in the different groups were

enumerated after 19 days in each case. Experiments were repeated 3 times with 6 mice per experiment. W/T= wild-type control mice. CCR5KO=CCR5 knockout. *Wild-type (purple), Wild-type non-infected (blue), CCR5KO (Aqua), CCR5KO non-infected (yellow)*

Table 1. Average Number of Pups in CCR5 KO vs. Wildype during Genital Chlamydia Infection

Animal Groups	2 wks post-infection	5 wks post-infection
CCR5 KO	8 ± 2.1	6 ± 1.4
Wildtype	3 ± 1.9	2 ± 3.3
Non-infected	7 ± 4.2	10 ± 0.9

±S.D

Immunological correlates of clearance of infection and protection from disease. The direct immunobiologic impact of CCR5 deficiency includes the limitation of T cell activation and recruitment of leukocytes to inflammatory sites of infection (Monno *et al.* 2001; Rockey *et al.* 2000). Since these processes are mediated by chemokines and cytokines, their production by WT and CCR5KO leukocytes that are exposed to chlamydiae, was evaluated. When splenic cells containing T cells and other leukocytes were exposed to chlamydiae, the antigen-specific TNF-α response was expectedly elevated in the cells from the WT mice (Fig. 29). The results presented in Figure 30 also revealed that the level of antigen-specific IFN-γ secreted by leukocytes from CCR5KO mice was not statistically different from the levels secreted by leukocytes from non-infected mice, indicating that that CCR5 is required for adequate activation of Th1 response against chlamydia. When the levels of the inflammatory chemokines RANTES

and IP-10 secreted by chlamydial exposed leukocytes from infected CCR5KO and WT mice were compared, it was also found that the knockout mice exhibited a diminished capacity (Figures 31 and 32), suggesting the deficiency of CCR5 results in a compromised Th1 response.

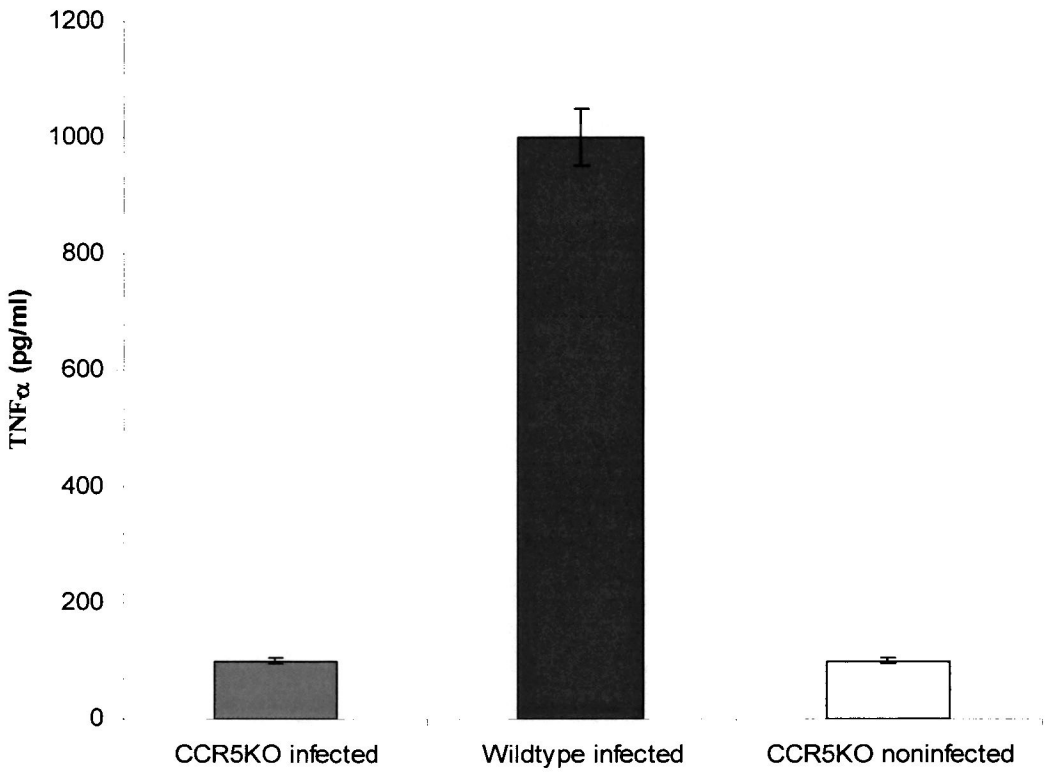

Figure 29. Measurement of TNF-α production in CCR5 KO mice during genital Chlamydia infection. Chemokine receptor knockout and control mice were genitally infected with MoPn. At 2 weeks post infection splenic cells were isolated from infection mice and 2 x 10^6 cells were stimulated with 10 ug/ml of chlamydial antigen for 120 h. The supernatants were collected and assayed for TNF-α. The absence of CCR5 decreased the amount of TNF-α produced.

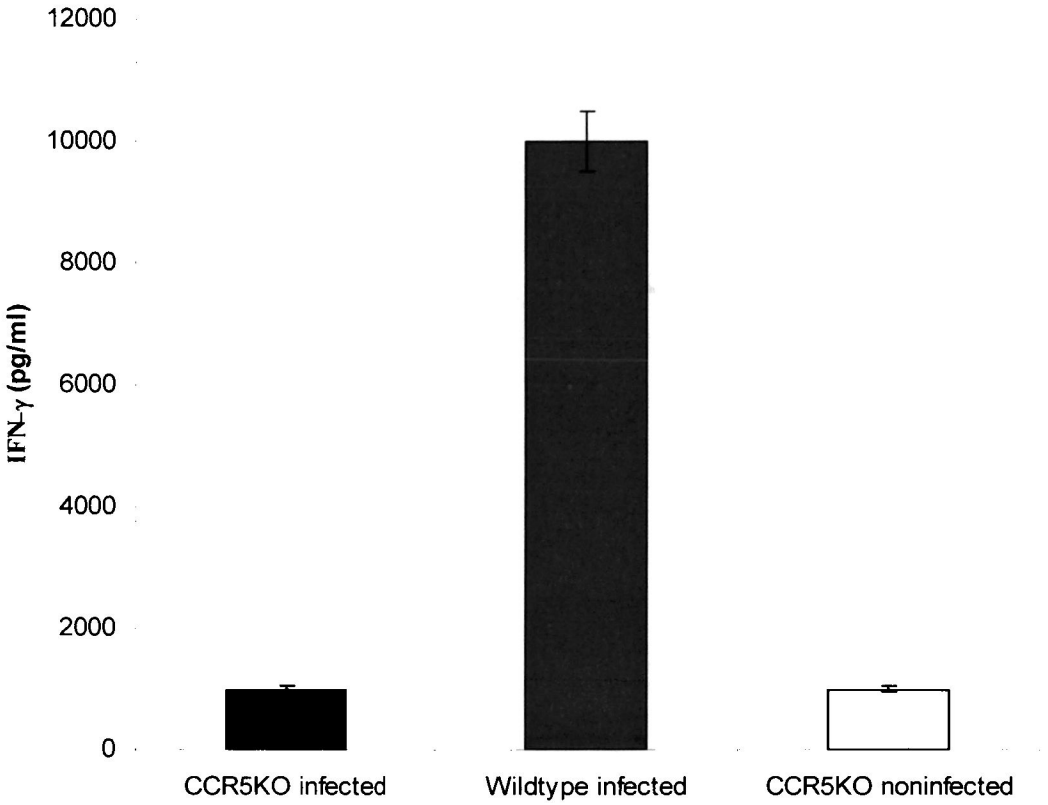

Figure 30. Measurement of IFN-γ production in CCR5 KO mice during genital Chlamydia infection. Chemokine receptor knockout and control mice were genitally infected with MoPn. At 2 weeks post infection splenic cells were isolated from infection mice and 2 x 10^6 cells were stimulated with 10ug/ml of chlamydial antigen for 120 h. The supernatants were collected and assayed for IFN-γ. The absence of CCR5 decreased the amount of IFN-γ produced.

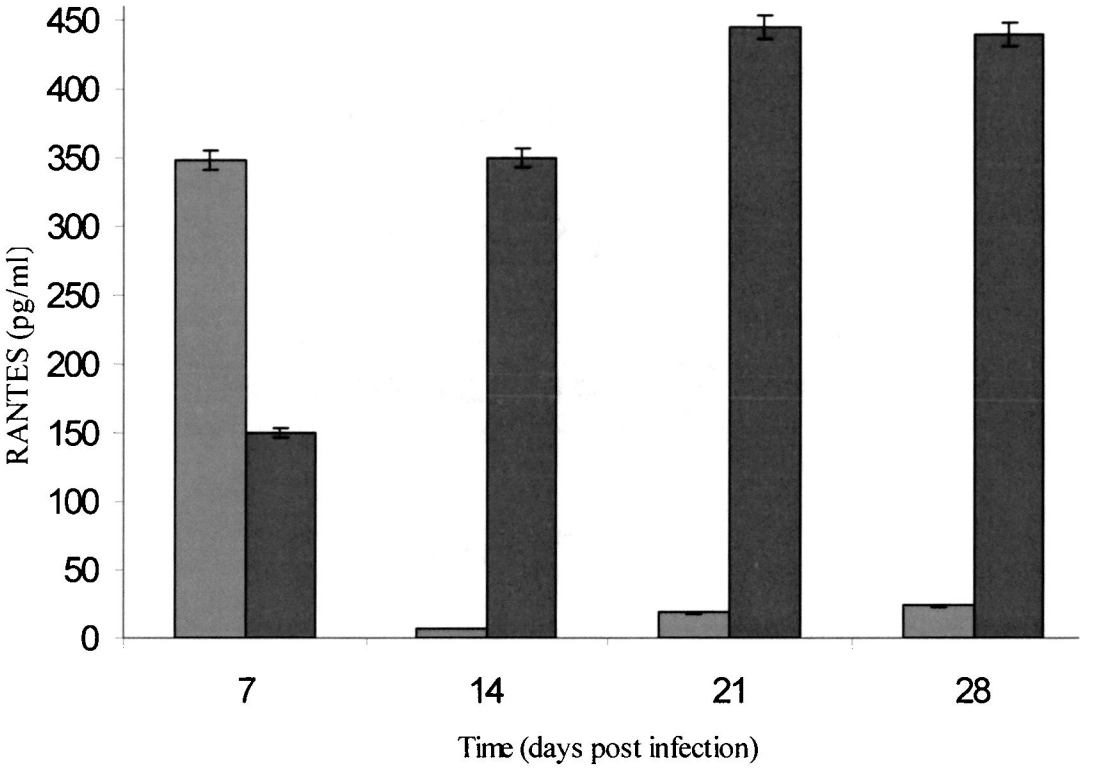

Figure 31. The production of RANTES in CCR5 KO mice during genital chlamydia infection. Chemokine receptor knockout (CCR5KO) (*blue*) and control mice *(purple)* were infected with *Chlamydia muridarum* (the *C. trachomatis* agent of mouse pneumonitis). At various times post-infection (7, 14, 21 and 28 days) splenic cells were isolated from infected mice and 2 x 10^5 cells were stimulated with 10 μg/ml of chlamydial antigen for 120 h. The supernatants were assayed using ELISA for RANTES.

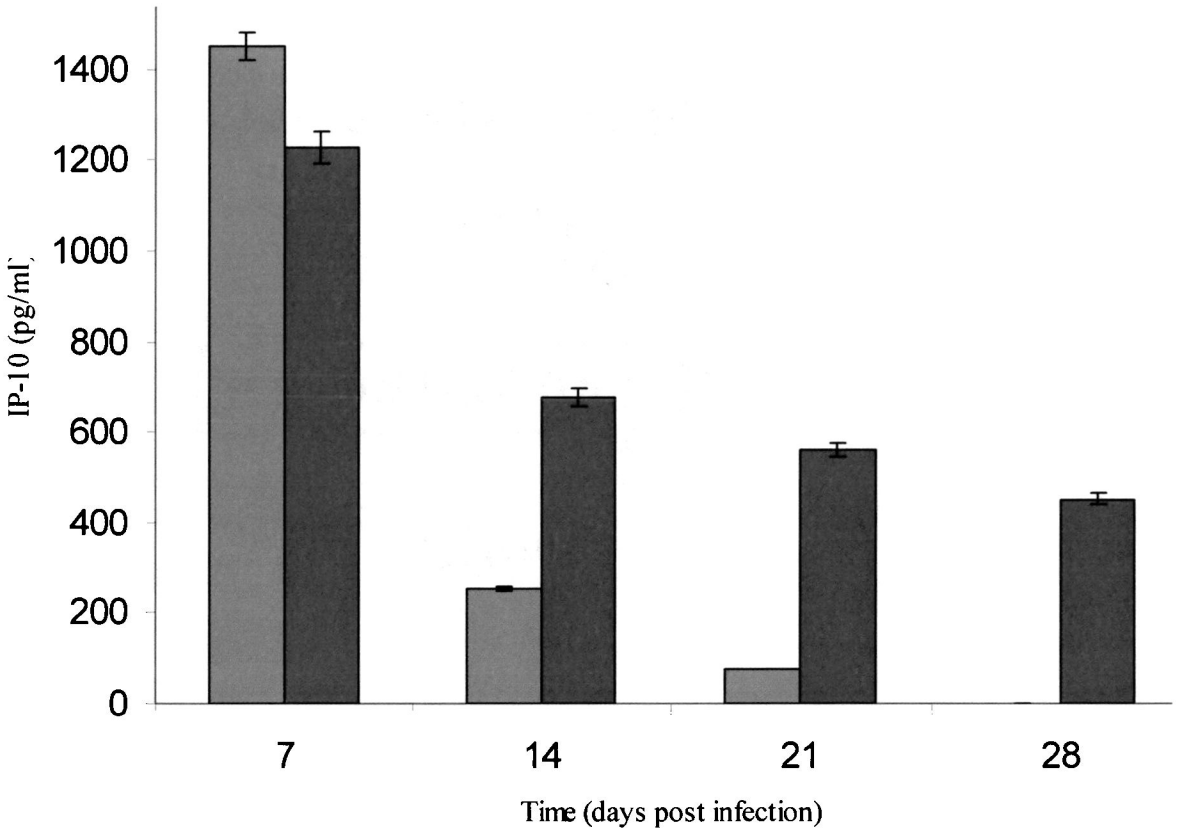

Figure 32. The production of IP-10 in CCR5 KO mice during genital chlamydia infection. Chemokine receptor knockout (CCR5KO) and control mice were infected with *Chlamydia muridarum* (the *C. trachomatis* agent of mouse pneumonitis). At various times post-infection (7, 14, 21 and 28 days) splenic cells were isolated from infected mice and 2 x 10^5 cells were stimulated with 10 μg/ml of chlamydial antigen for 120 h. The supernatants were assayed using ELISA for IP-10.

CHAPTER 5

DISCUSSION

The pathological consequences of genital infection by *Chlamydia trachomatis* include major sequelae, such as pelvic inflammatory disease and infertility. The urgent need to develop an efficacious vaccine poses a major clinical and basic research challenge of defining the requirements for inducing and maintaining protective genital mucosal immunity. Recent studies of animal models indicated that rapid and early elicitation and recruitment of certain immune effectors (specifically dendritic and Th1 cells) into local genital mucosae are crucial for antichlamydial immunity (Khamesipour *et al.* 1994; Su *et al.* 1995; Igietseme 2003). Thus, strategies for the definition of novel approaches to optimize the induction, recruitment, and retention of chlamydia-specific Th1 cells in genital mucosae are needed.

In general, the mucosal immune response to a vaccine is influenced by many factors, such as the antigen, vector, adjuvant, route of delivery, and hormones associated with the estrous cycle (for genital mucosal response) (Grayston 1962). It is conceivable that these factors affect antigen uptake, mucosal immune induction and homing recruitment, and retention of immune effectors in infected sites. Chemokines are important mediators of leukocyte trafficking and of the controlled recruitment of specific lymphocyte clonotypes during immune induction and inflammation. Epithelial cells and certain other cell types comprising the innate immune effectors elaborate a

number of cytokine and chemokines upon encountering mucosally routed antigens (Amigorena *et al.* 1999; Guyre *et al.* 1997). Presently unknown, various factors may regulate the expression and functions of certain chemokines, chemokine receptors, and adhesion molecules. Sex hormones have been shown to regulate the immune responses in the female reproductive tract (McMurray 2001). The estrous cycle and treatment with sex hormones such as estradiol and progesterone, have been demonstrated to influence mucosal immune elicitation and function in the genital mucosal surface, cytokine expression and recruitment or trafficking of leukocytes into the reproductive tract (McMurry 2001). Moreover, epidemiologic and biologic evidence suggests that infection with *C. trachomatis*, the leading cause of nongonococcal PID, is enhanced by oral contraceptives (Washington 1985). The hypothesis investigated in the present study was that estrogen alters the expression of immunoregulatory factors such as chemokines, thereby affecting their roles in the induction and recruitment of leukocytes during Chlamydia infection. The results revealed that estrogen suppressed the expression of certain chemokines responsible for recruiting the relevant Th1 and dendritic cells into genital mucosae.

Studies in the mouse as well as other animal models have shown that hormones play an important role in controlling immune responses to chlamydial infection and in determining the outcome of infection (Palsey 1985; Rank 1987). In general, mouse models require pretreatment with progesterone prior to exposure, to enhance infections, especially with human serovars (Kaushic 2000). Others have shown that in the absence of progesterone pretreatment, establishment of infection is dependent on stage of the estrous cycle or requires high infectious doses (Bose and Goswami 1986). Guinea pigs

do not require progesterone and are easily infected with *C. psitacci*, but develop heavier infection following estradiol treatment (Rank 1987). A basic understanding of the mechanism by which estradiol is increasing chlamydia infectivity is greatly needed.

In vitro studies indicated that the pretreatment of estrogen causes an increase in inclusion development (Fig. 4). These results confirmed earlier results by others indicating that pretreatment of estrogen causes an increase in attachment and inclusion development in HeLa cells and McCoy cells (Bose and Goswami 1986). Furthermore, Wyrick and colleagues (2000) studies revealed that attachment of *C. trachomatis* is approximately 50% in human endometrial gland epithelial cells. Addition of estrogen (10^{-10} M) to the culture medium enhanced chlamydial attachment to human endometrial gland epithelial cells to approximately 80%. Understanding this increase in chlamydia infectivity in cultured cells is of great interest. Recent studies have shown that protein disulfide isomerase, a component of the estrogen receptor complex is associated with *C. trachomatis* serovar E attachment to human endometrial epithelial cells (Wyrick 2000). Localization studies performed by Sugarman (1986), suggested that the effect of estrogen on the infectivity of *C. trachomatis* is dependent upon initial interactions of estrogens with McCoy cells. In the same study, light and electron microscopy of the McCoy cells showed no morphological changes after exposure to estrogen under the incubation conditions. Estrogen may modify host susceptibility to chlamydia infection in a manner independent of morphological changes in mammalian cells. These findings are of importance because in an *in vivo* model, infection of epithelial cells causes initial action of the innate immune response by the secretion of chemokines. Expression of chemokines within tissues regulates the recruitment of specific subsets of lymphocytes to

distinct tissue sites. Chemokines are therefore responsible, in part, for directing the immune response that ensues following bacterial invasion. Epithelial cells play a central role in directing the immune response, since they host Chlamydia and secrete cytokines, such as IL-8, early after infection. The importance of the role these cells play in the secretion of chemokines, led to our interest that estrogen may affect the production of chemokines following Chlamydia infection. These data showed that pretreatment of estrogen significantly decreased the production of MIP-1α, RANTES and MCP-1 in McCoy cells (Figs. 5-15). Interestingly, these are the first reported findings that indicate estrogen's effect on the expression of these chemokines in chlamydia infected cells. These studies support other findings that determined the regulatory roles of sex hormones in cutaneous biology and immunology in the skin. Studies indicated that estrogen *in vitro* down-regulates the production of the neutrophil type 1 T cells and macrophage-attracting chemokines, CXCL8, CXCL10, CCL5, and RANTES by keratinocytes (Kanda 2003).

Findings of this investigation, led to the hypothesis that if estrogen causes a decrease in the secretion of some of these chemokines *in vitro* then there would be a similar trend in an animal model. This decrease could possibly have an effect on the activation and recruitment of T lymphocytes to the site of infection, thereby affecting the induction of protective immunity. Wild-type mice were treated for seven days with 10^{-8} M (10-fold higher than the physiological concentration) in mice followed by chlamydia infection. Estrogen delayed the clearance of chlamydia compared to the wild-type (Fig. 16). These results are in accord with Rank and colleagues (1985) who suggested that estradiol treatment increased the apparent number of infected cells in the cervix and vagina. The chemokine profile data collected during this *in vivo* study supported the *in*

vitro findings, indicating that estrogen caused a decrease in MIP-1α at 7, 14, 21, and 38 days (Fig.17). The most obvious difference was observed at 21 days post-infection which proves to be significant. According to some previous studies, approximately 14 to 21 days post-infection, there is a high expression of the CCR5 receptor in the T cells of the genital tract of chlamydia infected mice. Similar findings were observed in the MIP-1β and RANTES (Figs. 18 & 19). The production of RANTES was more significant at 21 days post-infection. The obvious increase in production of these chemokines at 21 days post-infection have also been observed in other studies performed in the lab by Belay *et. al* (2002). These studies indicated the expression of RANTES, MCP-1, MIP-1α, IP-10 early in infection. Interestingly, there was a robust production of RANTES in the estrogen treated animals around 7 days post-infection. The explanation for this response is unknown. It could possibly be attributed to a high nonspecific response or could be due to factors that are to be determined in future studies.

The role of estrogen in the regulation of the inflammatory response is not well defined as it relates to chlamydia infection. It was observed that estrogen decreased the production of inflammatory cytokines IL-6, IL-1α, and TNF-α (Figs. 22-24). These results could be significant because of the correlation between chlamydia infection and PID which results from inflammatory response (Barr *et al.* 2005). The immune response to genital chlamydial infection is very complex: it clears infection and confers short term protection but at the same time sensitizes the host for development of immunopathological changes (Kaushic *et. al* 2005). Williams and colleagues (1998) showed that IL-6 KO mice had significantly increased *C. trachomatis* levels in lung tissue and increased mortality compared to wildtype controls early after intranasal

infection. Other studies have shown that IL-1α and TNF-α which play indirect roles in leukocyte recruitment by upregulating ICAM-1, E-selectin and VCAM-1 on adjacent endothelia (Lovdal *et al.* 2000). Moreover, TGF-α, TNF-α and large quantities of IL-1α and IL-6 have been associated with scarring and fibrosis in animal models and humans (Lanzavecchia 1996; Lovdal *et al.* 2000). This information raises the possibility that Chlamydia-infected epithelial cells directly contribute to scarring of the fallopian tubes. (Johnson 2004)

In view of the fact that estrogen decreases the production of inflammatory cytokines, there may be a positive effect on the immunopathology that is usually associated with infertility. When this hypothesis was tested, surprisingly, estrogen did not have an effect on the pregnancy rates during Chlamydia infection. These studies are supported by other studies that showed oral contraceptives did not affect the duration of shedding of organisms nor the histopathology of acute chlamydial infection of the reproductive tract (Kaushic *et al.* 2000). The results suggest that although estrogen decreases inflammatory responses, there is not a significant effect on the immunopathology that is usually associated with the influx of inflammatory cells (Fig. 26). There may be other mechanisms by which infertility is occurring. Further studies could possibly investigate the effect of pre-treatment and post-treatment of estrogen on the effects of pregnancy. Ongoing experiments in the laboratory are studying the effects of pregnancy on chlamydia infection. Others have evaluated the effect of *C. trahomatis* on pregnancy which resulted in low birth weight births and/or stillbirths (Rastogi 2003), Lawton *et al.* 2004).

Similar studies have shown that estrogen treatment down-regulates TNF-α production and reduces the severity of experimental autoimmune encephalomyelitis in cytokine knockout mice (Ito *et al.* 2001). Studies have indicated that estrogen treatment may possibly cause a shift to Th 2 production; however, that was not the case in this study.

In addition, estrogen had a significant effect on GM-CSF (granulocyte macrophage colony stimulating factor) secretion which is responsible for the maturation of dendritic cells which act as antigen presenting cells (Fig. 21). GM-CSF is produced by T cells, B cells, macrophages, mast cells, endothelial cells, neutrophils, eosinophils, and fibroblasts, when these cells are stimulated by certain cytokines or other factors during inflammation. GM-CSF is a pleiotropic cytokine that promotes the proliferation, maturation, and activation of different hematopoietic cells at various developmental stages. This could prove to be significant because earlier studies have indicated that estradiol regulates antigen presentation by vaginal cells and that vaginal cells, in turn, influence antigen presentation, as well as B and T cell proliferation (Wira *et al.* 2000).

Estrogen decreased ICAM-1 production (Intercellular adhesion molecule type-1) in the genital tract of chlamydia infected mice (Fig. 20). These findings are significant because earlier studies in the laboratory of Joseph Igietseme revealed that ICAM-1 showed a pattern of expression in the genital tract of noninfected control mice and chlamydia infected mice (Belay *et al.* 2002). The expression followed the expression patterns of Th1-related chemokines, peaking by day 21 and appearing to be reduced by day 42 (Belay *et al.* 2002). Adhesion molecules enhance adherence of the T cell to the APC/target cell transmembrane proteins or glycoproteins. Adhesion molecules on one

cell interact with complementary adhesion molecules on the other cell. T cell activation is imperative in the clearance of chlamydia. These results might suggest that estrogen may act as an immunosuppressor by decreasing the production of ICAM-1 which would in turn interfere with antigen presentation and or T cell activation. Further studies are needed in order to determine the mechanism by which estrogen may be effecting the expression of this molecule. Earlier findings have suggested that one of the mechanisms by which estrogen decreases parthogenesis is by the suppression of vascular cellular adhesion molecule-1 (VCAM-1) expression, which leads to decreased macrophage recruitment to the arterial wall early in the process. (Seli *et al.* 2002).

The effects of estrogen on infectivity or immune response toward foreign agents is not unambiguous for chlamydia, but other bacteria causing diseases such as gonorrhea have been of great concern for those who are on oral contraceptives. In mice treated with estradiol, bacteremia progressively developed within 12h post inoculation and mice died within the next 6 h. The administration of estradiol affected by the function of polymorphonuclear leukocytes (PMN) responsible for eliminating gonococci, but the progesterone did not (Kita *et al.* 1981).

As mentioned earlier, estrogen had a significant effect on the production of RANTES, MIP-1α and MIP-1β. These chemokines are ligands with high affinity for CCR5. Interestingly, recent findings indicate that chemokine receptors such as CCR5 act as co receptors (with the CD4 molecule) for entry of the human immunodeficiency virus (HIV). In other similar studies, estrogen treatment significantly decreased expression of proteins corresponding to the chemokine receptors CXCR3 and CCR5 on mammary cells. Results suggested that exposure of mammary tissue to estrogen may decrease the

release of local chemokines from mammary cells; potentially increasing the risk of tumor growth through decreased immune surveillance (Aronica *et al.* 2004).

For the aforementioned reasons, chemokine receptor-5 was of particular interest in our study. We chose to determine its role in the induction of protective immunity against chlamydia. Studies have shown that CCR5-deficient mice were significantly more susceptible to infections with the parasites *Listeria monocytogenes*, *Cryptococcus neoformans*, and *Toxoplasma gondii*. They also showed increased mortality to influenza A virus infection (Mack *et al.* 2001). Belay *et al.* (2002) found that T cells expressed CCR5 during genital chlamydia infection. This led to the hypothesis that CCR5 may play a vital role in the induction of protective immunity. In this study, we have experimentally demonstrated that the CCR5-related inflammatory response is crucial for the development of tubal factor infertility (TFI) following genital chlamydial infection. In translational immunogenetic and pathobiologic clinical studies in humans, functional defect in CCR5 also appears to moderate the development of tubal pathogenesis associated with genital chlamydial infection in women. These data are corroborated by previous propositions that certain host factors are relevant for the development of the complications of chlamydial infection. Specifically, the 32 bp deletion in the CCR5 gene, which results in a truncated protein with impaired signal-transduction capacity, was associated with resistance to human immunodeficiency virus type 1 (HIV-1) infection (Kinnunen *et al.* 2003; Dean *et al.* 1996). Recently, it has been suggested that heterozygosity for CCR5delta32 was also associated with spontaneous hepatitis C viral clearance and with significantly lower hepatic inflammatory scores (Goulding *et al.* 2005). These experimental studies indicated that a deficiency of specific antichlamydial

Th1 response led to a suppression of Th1 response and delayed clearance of genital chlamydial infection. However, CCR5KO mice were protected from the complications of the genital infection relating to infertility (Fig. 28). In addition, *C. trachomatis*-exposed women with CCR5delta32 deletions appear to be protected from tubal pathology as well, suggesting a crucial role for CCR5-related specific Th1 and inflammatory responses in the pathogenesis of infectious tubal pathologies. Similar findings have shown that the lack of CCR5 reduced the number of ulcerations in dextran-sulfate-mediated colitis and increased the tolerance to LPS (Mack *et al.* 2001).

Perhaps this study represents the first concurrent demonstration of a strong causal relationship between CCR5-related specific Th1 and inflammatory response and development of complications of genital chlamydial infection in both animal models and humans. The implications include the fact that although Th1 response is crucial for chlamydial control, there are host conditions that could skew the response toward pathology. Such conditions may include the involvement of immunopathogenic chlamydial antigens (LaVerda *et al.* 1997) and vaccine design effort may focus on defining the existence of clonotypic T cells that recognize such antigens or develop additional strategies to eliminate them from promising vaccine candidates. T cell clones reactive against specific chlamydial antigens have been isolated from the synovial fluids of patients suffering from chlamydia-induced reactive arthritis (Hassel *et al.* 1993). In addition, since an early and relatively robust T cell response is protective against subsequent development of complications of chlamydial infection (Igietseme 2003), these results from the CCR5KO system may suggest that the lack of an early T cell activation caused the delay in resolution of the infection; however, the persisting suppression of T

cell activation prevented the chronic host inflammatory response that induces pathologies. Therefore, an early treatment of chlamydial infection with antimicrobials followed by specifically targeted anti-inflammatory agents may hold promise as a strategy for preventing the complications of chlamydial infection. Finally, it is pertinent to mention that although previous studies along this proposition yielded conflicting results, the selective use of specifically targeted anti-inflammatory agents in combination with antibiotics could prove useful in the management of chlamydial infections to avert pathology. In fact, inflammatory processes induced by several species of Chlamydia could be suppressed by certain non-steroidal anti-inflammatory drugs, including aspirin and indomethacin.

In summation, this study has revealed novel results indicating the influence of estrogen and CCR5 on the immune response against chlamydia. This is the first study to show the effect of estrogen on inflammatory cytokine production during genital chlamydia infection. These findings may be helpful in developing therapeutic treatments protecting infected women from the development of inflammation that is associated with PID. In addition, the role of Th1 chemokines/chemokine receptor-5 proved to be vital in their ability to activate and recruit T lymphocytes to the site of infection. These data could be useful in the designing and development of a vaccine. Modulation of specific chemokines and their specific receptors (i.e. CCR5 and CXCR3 for Th1 and CCR3 for Th2) can therefore be useful in promoting the induction and recruitment of Th1 cells into the genital mucosa to ensure protective immunity against Chlamydia (D'Ambrosio *et al.* 2000). Although promising, the impact of conditionally expressed chemokines as an adjuvant system in a vaccine construct to modulate mucosal immune response against

Chlamydia has yet to be determined. However, despite the presence of chemokines and $CD4^+$ Th1 cells in the upper genital tract, infected mice still suffer ascending infection, suggesting that the immune response elicited in the upper genital tract remains insufficient to control the spread of chlamydiae from the lower to the upper genital tract (Belay *et al.* 2002; Maxion and Kelly 2002).

To date, scientists have developed recombinant multi-subunit vaccines using chlamydia antigens (OMP1 and PORB) (Eko *et al.* 2002). Various adjuvant systems have been used and presently in our laboratory we have been evaluating the use of recombinant technology to engineer Lactobacillus as a potential delivery vehicle for chlamydia antigen(s) and adjuvant system. By including gene sequences for chemokines such as RANTES, MIP-1α and IP-10 in a recombinant plasmid may prove useful in the induction, recruitment, and retention of chlamydia-specific Th1 cells in genital mucosae. Further studies are needed to evaluate the immunogenicity and protective properties of such a vaccine.

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